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(54) Title: USES OF FLAVIVIRUS RNA–DEPENDENT RNA POLYMERASES

(57) Abstract

An isolated recombinant viral polymerase is provided that is useful in diagnostic and anti–viral compound screening applications.

USES OF FLAVIVIRUS RNA-DEPENDENT RNA POLYMERASES

Background of the Invention

Viruses are obligate parasites which depend upon the infected host for many of the basic processes needed for a successful infection. Because viruses depend on the enzymatic and synthetic functions of the host cell, it is very difficult to treat viral infections without affecting cellular processes. Given that several viral diseases are at pandemic proportions, including influenza, AIDS, and hepatitis, the design of effective virus-specific drugs is increasingly important.

For example, potential antiviral drugs are screened to determine whether the drug can preferentially inhibit a viral process. One such drug is Aziduovir, which is utilized more readily by the reverse transcriptase of the human immunodeficiency virus (HIV) than by host cellular polymerases. However, Aziduovir and other viral inhibitors were generally discovered through an intensive and costly drug screening program.

Other antiviral treatments, including interferon, cause significant and widespread changes in the cell and hence lead to a number of side effects, including fever, nausea, and other discomfort. Yet other potential antiviral therapies, including antisense molecules and ribozymes, are difficult to produce due to the need for complex molecular recombinant technology.

Viral RNA replication, a process fundamental to viral pathogenicity, requires specific recognition of RNA features by proteins. RNA-dependent RNA polymerase (RdRp) is a complex composed of viral and cellular proteins that directs viral RNA synthesis from infecting RNA templates. Although the sequences of many viral RdRp proteins have been determined and analyzed, few of the mechanistic details of RNA-dependent RNA synthesis, a process fundamental to viral pathogenicity, have been elucidated. Consequently, knowledge of RdRps is significantly less than that of other RNA and DNA polymerases.

Initiation of viral RNA synthesis provides an ideal target for antivirals directed toward viral RNA replication. Studies of transcription, translation and

and Schlesinger, 1996, which is incorporated by reference herein). As described hereinbelow, the recombinant RdRp of bovine viral diarrhea virus initiates *de novo* RNA synthesis. Thus, recombinant RdRps are useful to screen agents that specifically inhibit *de novo* initiation of RNA synthesis. Moreover, the recombinant RdRp may also be useful in diagnostics.

Yet another embodiment of the invention is a method to detect viral RdRps that initiate *de novo* RNA synthesis. The viral RdRps preferentially initiate *de novo* RNA synthesis over elongative RNA synthesis, i.e., elongative RNA synthesis requires a primer, e.g., a 3' hydroxyl group from a template nucleic acid or from an oligonucleotide primer. The method comprises:

(a) contacting a nucleic acid template with an isolated recombinant viral RNA-dependent RNA polymerase under conditions effective to initiate *de novo* RNA synthesis so as to yield a nucleic acid product; and

(b) detecting or determining the presence of the nucleic acid product.

The nucleic acid template may be RNA, DNA, a chimera of RNA and DNA, or contain modified nucleotides. The nucleic acid product is preferably a RNA product. However, the nucleic acid product may comprise modified nucleotides. As used herein, modified nucleotides includes nucleotides that are modified in the sugar, base and/or phosphate moiety of the nucleotide, which modification does not substantially impact the incorporation of the modified nucleotide into the nucleic acid product, e.g., the modified nucleotide is incorporated into the nucleic acid product at least about 0.01%, preferably at least about 0.1%, and more preferably at least about 1%, that of a naturally occurring nucleotide. For example, modified nucleotides include those which are labeled, for example, with radioisotopes or fluorescent moieties.

Also provided is a method to identify agents that specifically inhibit *de novo* initiation of RNA synthesis. The method comprises:

(a) contacting the agent with a mixture comprising an isolated recombinant viral RNA-dependent RNA polymerase and a nucleic acid template under conditions that result in *de novo* initiation of RNA synthesis so as to yield a RNA product; and

combined with the recombinant viral RNA-dependent RNA polymerase and the viral nucleic acid in the sample, results in *de novo* initiation of RNA synthesis. Preferably, the mixture comprises nucleotides, e.g., ribonucleotide triphosphates or modified nucleotides. Preferably, the modified nucleotide is detectable.

Brief Description of the Figures

Figure 1. BMV RdRp accurately initiates RNA synthesis from RNA or DNA proscripts. (A) Proscript -20/13, complementary to the viral (+)-strand RNA3 from positions 1222 to 1252, contains the WT BMV subgenomic promoter directing the synthesis of a 13-nt product and serves as the WT control. The initiation nucleotide is denoted by an arrow with the sequence of the RdRp product shown above. Schematics of various constructs tested are listed and to the right are the lane numbers showing the amount of RNA synthesis relative to that from the WT control in the corresponding autoradiograph. RNA sequences are denoted by bold capital letters while DNA sequences are in lower-case letters. Nucleotide substitutions in both RNA and DNA constructs are shown below each sequence. The Δ -1 g proscripts lack the 3′ terminal guanylate at position -1 relative to the initiation site. (B) Autoradiographs of BMV RdRp reaction products. The amount of RNA synthesis from 25 nM of proscript 20/13 WT is shown in lanes 1 and 10. T7 generated markers containing the expected sequence of the RdRp products were used to determine the sizes of the accurately initiated 13- and 14-nt BMV RdRp products. The 14-nt product is due to a 1-nt nontemplated addition by RdRp. Reactions using 25 nM of the all deoxyribose proscript, d(-20/13), are shown in lanes 2-9. RNA synthesis and accurate initiation from proscript d(-20/13) were verified by the treatments indicated above the gel in lanes 3-5 and lanes 6-9, respectively. The amount of RdRp product from 125 nM of RNA or DNA templates with a penultimate initiation site is shown in lanes 11-21. The treatments shown above lanes 12-14 and 19-21 demonstrate the initiation requirements from r(-1/13) and d(-1/13), respectively. The treatments indicated above lanes 16-18 verify RNA synthesis from d(-1/13). Lane ϕ represents the products of a control reaction with no added template while Std lanes represent products with no additional treatments.

Figure 2. Ribose moieties which facilitate RNA synthesis by RdRp. (A) The sequence of the -20/13 WT proscript is shown with the initiation site marked

Rev proscript serves as a negative control. The names and I_{50} values are listed to the sides. (B) Determination of I_{50} values for the DNA inhibitors. The amount of 15-nt product generated from the -20/15 RNA proscript was measured in the presence of increasing amounts of DNA templates. The I_{50} value was quantitated as the concentration of inhibitor needed to reduce the 15-nt product from 25 nM of -20/15 proscript by 50%. The identities of the inhibitors are shown to the right of the graph. Data points represent the mean of three independent experiments with deviations shown.

Figure 5. Synthesis of positive strand RNA using DNA templates. A) Template d(-1/13) containing the sequence complementary to nucleotides 1241-1252 of BMV RNA3 are shown with the initiation cytidylate indicated by an arrow. Changes to d(-1/13) focusing on the 3' end, the +2 adenylate, the +3 uridylate and the +4 adenylate were used for RNA synthesis by BMV RdRp. The changes are indicated above the autoradiogram of the RdRp products. The positions of the 13 and 14 nucleotide products are shown on the left. The reaction products were separated by denaturing 12% PAGE and visualized by autoradiography. B) Summary of the effect of nucleotide changes focusing on the 3' end of the initiation site. All results presented were from at least three independent trials. C) Summary of the effect of nucleotide changes at positions +2, +3, and +4 from d(-1/13). D) Effect of changes to a guanylate in the first six positions in d(-1/13) on positive strand RNA synthesis.

Figure 6. Effect of nucleotide changes in d(-1/13) on the ability of the resultant DNA template to compete for RdRp. An RNA template r(-20/15) directing synthesis of a 15-nucleotide product was used as a reference. The amount of RNA synthesis generated in the presence of the different templates are listed as a percentage compared to the amount of synthesis from r(-20/15) without any competitor. Competition assays were carried out in the presence of five and ten fold excess of the competitor DNA. All results were from at least three independent trials. ND, not determined.

Figure 7. Concentration of several DNA templates needed to reduce RNA synthesis from r(-20/15) by 50%. Percent synthesis from r(-20/15) directing synthesis of a 15-nucleotide product was measured in the presence of

different endscripts are given as a percentage relative to B2(-)46G. The results presented are from three independent trials.

Figure 10. Effect of multiple initiation sites on RNA synthesis. The authentic initiation site is indicated by an arrow in the first construct marked 1. Additional initiation sites added to the 3' end of B2(-)46G are indicated by arrows 2 and 3. The three initiation sites should generate products of 46-, 49-, and 52-nucleotides. RNA synthesis directed by the different initiation sites from their respective templates are presented relative to initiation from cytidylate #1 in B2(-)46G. Products initiated from the three potential initiation sites are indicated on the right as 1, 2, and 3 respectively.

Figure 11. Requirements for positive strand RNA synthesis. A) The predicted secondary structure of B2(-)46G with the stems (A1 and A2) and loops (L1 and L2) indicated by brackets. The initiation cytidylate is noted by an arrow. B) Deletion analysis of the predicted stem-loop region. The templates used in the specified reactions are indicated at the top of the autoradiogram. Endscripts that have a cytidylate at the +1 position are initiation-competent and are indicated by "+", while initiation–incompetent endscripts are indicated with a "(". The sizes of the RdRp products are denoted on the right. The amount of positive strand synthesis directed by B2(-)(3-11 and B2(-)(17-26 were 200% and 70% respectively as compared to B2(-)46G after correcting for the number of CMP incorporated. The results presented are an average from three independent trials. C) Deletion and further analysis of sequences required for efficient RNA synthesis. The templates used and whether they can direct initiation of RNA synthesis (+ or () are indicated on the top of the autoradiogram. The sizes of the RNA products are denoted on the right side of the autoradiogram. The amount of synthesis, after adjusting to the number of radiolabelled CMP incorporated, from B2(-)26G, B2(-)26TV, B2(-)22G, and B2(-)16G were 100%, 17%, 22%, and 5% respectively. D) Alignment of the sequences in B2(-)26G, B2(-)(3-11 (deletion of A2 stem region), B2(-)(17-26 (deletion of L1 loop region), and B2(-)26TV (transversion of nucleotide sequence 17-24). The two guanylates as well as the two adenylates present at the 5' end are denoted in bold letters.

Figure 12. A model for the interaction between the BMV RdRp and the minus strand endscript required for initiating genomic positive strand synthesis.

Figure 14. *De novo* initiation of RNA synthesis from DNA templates. The ribose or deoxyribose forms of (-)21g used in the reactions are indicated above the lanes of a 20% denaturing polyacrylamide gel. DNA +1C/G was used to demonstrate that all initiation from a DNA template takes place from the +1 cytidylate. The position of the 21-nt product is indicated to the right.

Figure 15. Template nucleotides required for efficient RNA synthesis by NS5B. A) RNAs used in the reactions. The names of the RNAs used are shown to the left, followed by the relevant nucleotides. "-" denotes that a nucleotide was deleted. Nucleotides in small bold letters are non-BVDV sequences added to the 3′ end of the template. Nucleotides in capital bold letters were changed from the wild-type BVDV sequence. B) Autoradiogram of a 20% denaturing polyacrylamide gel of the products made by NS5B using the template indicated above the lanes. The symbol ϕ denotes a reaction performed in the absence of exogenous templates. All of the lanes used in the figure were from performed in one experiment, but they were sectioned to allow more coherent description of the results. The position of the 21-nt product is denoted on the right.

Figure 16. Template nucleotides required for recognition by NS5B. A) Autoradiogram of a denaturing polyacrylamide gel showing the results of a competition experiment. The 27-nt product and the higher molecular weight products are the products from template 3-init. The 21-nt product, where present, was generated from the competitor RNAs, whose identity is indicated above the autoradiogram. All reactions shown were performed with 20 pmoles of competitor in triplicate to allow quantification. B) Quantification of the amount of products synthesized from RNA 3-init in the absence or presence of competitor RNAs. C) A model summarizing the key features in the interaction between NS5B and the 3′ end of the template RNA.

Detailed Description of the Invention

As the structures of several different polymerases appear conserved, it has been suggested that the mechanism of nucleic acid synthesis is also conserved (O'Reilly and Kao, 1998). The best characterized polymerases are the DNA-dependent RNA polymerases (DdRp) that are responsible for transcription. DNA-dependent RNA synthesis has been divided into a number of biochemically distinct steps: binding of the DdRp to the promoter, formation of a

whereas the T7 RNA polymerase remains more stably bound to supercoiled DNA, although the stability of the T7 RNA polymerase-DNA interaction is highly dependent on the structure of the template (Diaz et al., 1996). Third, stability of the DdRp ternary complex is maintained primarily by RNA-protein and DNA-protein interactions, and not by RNA-DNA interactions (Altmann et al., 1994). For RdRp, it is possible that for some viruses, an intermediate of (−)-strand RNA synthesis is a double-stranded hybrid composed of the nascent and template RNAs (Baltimore, 1968; Takeda et al., 1986; Bienz et al., 1992; de Graaff et al., 1995). If true, then the duplex may contribute to the stability of RdRp ternary complex.

Two models to define the steps in RNA synthesis by RdRps for (+)-strand RNA viruses are recombinant RdRp, e.g., a recombinant Flaviviridae RdRp, or isolated viral replicase, which includes the RdRp. For isolated viral replicase, BMV is a prototype for the alphavirus superfamily. BMV has three genomic RNAs, designated RNA1, 2, and 3 and a subgenomic RNA4. These RNAs encode four proteins: the helicase-like 1a (109 kDA), the polymerase-like 2a (96 kDa), the movement protein 3a (34 kDa), and the capsid protein (20 kDa). Each BMV RNA contains a highly conserved 3′ region which folds into a tRNA-like structure that is required to direct the synthesis of (−)-strand RNA. The (−)-strand RNA serves as template and provides *cis*-acting sequences for genomic (+)-strand and subgenomic RNA synthesis.

The BMV RNA replication enzyme is a complex localized in the endoplasmic reticulum. It contains the BMV-encoded 1a and 2a proteins and yet unidentified host proteins. Membrane-associated replicase can be solubilized with nonionic detergents and still retain the ability to direct synthesis of (−)-strand RNAs or subgenomic (+)-strand RNA from exogenously added genomic RNAs or (−)-strand BMV RNA3, respectively. Detergent-solubilized BMV replicase, named RNA-dependent RNA polymerase (RdRp), can utilize (+)-strand RNAs of less than 160 nucleotides containing the conserved tRNA-like sequence to direct BMV-specific RNA synthesis *in vitro*.

template to be amplified. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, and the like. See generally Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51, 263 (1987); Erlich, ed., PCR Technology, (Stockton Press, NY, 1989).

Primers are made to correspond to highly conserved regions of polypeptides or nucleotide sequences which were identified and compared to generate the primers, e.g., by a sequence comparison of known viral RdRp sequences. One primer is prepared which is predicted to anneal to the antisense strand, and another primer prepared which is predicted to anneal to the sense strand, of a nucleic acid molecule which encodes a viral RdRp.

The products of each PCR reaction are separated via an agarose gel and all consistently amplified products are gel-purified and cloned directly into a suitable vector, such as a known plasmid vector. The resultant plasmids are subjected to restriction endonuclease and dideoxy sequencing of double-stranded plasmid DNAs.

As used herein, the terms "isolated and/or purified" refer to *in vitro* isolation of a nucleic acid or polypeptide molecule from its natural cellular environment, and from association with other components of the virus or cell, such as nucleic acid or polypeptide, so that it can be sequenced, replicated, and/or expressed. For example, "isolated viral RdRp nucleic acid" is RNA or DNA containing greater than 9, preferably 36, and more preferably 45 or more, sequential nucleotide bases that encode at least a portion of a viral RdRp, or a RNA or DNA complementary thereto, that is complementary or hybridizes, respectively, to RNA or DNA encoding the RdRp and remains stably bound under stringent conditions, as defined by methods well known in the art, e.g., in Sambrook et al., *infra*. Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the nucleic acid and is preferably substantially free of any other non-viral nucleic acid.

As used herein, the term "recombinant nucleic acid" or "preselected nucleic acid," e.g., "recombinant nucleic acid sequence or segment" or "preselected nucleic acid sequence or segment" refers to a nucleic acid, that has

contain coding regions flanked by control sequences which promote the expression of the preselected DNA present in the resultant cell line.

As used herein, "chimeric" means that a vector comprises DNA from at least two different species, or comprises DNA from the same species, which is linked or associated in a manner which does not occur in the "native" or wild type of the species.

Aside from preselected DNA sequences that serve as transcription units for a viral RdRp, a portion of the preselected DNA may be untranscribed, serving a regulatory or a structural function. For example, the preselected DNA may itself comprise a promoter that is active in insect cells, prokaryotic cells, or mammalian cells, or may utilize a promoter already present in the genome that is the transformation target. Any suitable promoter element may be employed in the practice of the invention.

Other elements functional in the host cells, such as introns, enhancers, polyadenylation sequences and the like, may also be a part of the preselected DNA. Such elements may or may not be necessary for the function of the DNA, but may provide improved expression of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the cell.

"Control sequences" is defined to mean DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotic cells, for example, include a promoter, and optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Operably linked" is defined to mean that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a peptide or polypeptide if it is expressed as a preprotein that participates in the secretion of the peptide or polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate

Transformation into Host Cells

The recombinant DNA can be readily introduced into the host cells, e.g., mammalian, bacterial, yeast or insect cells by transfection with an expression vector comprising DNA encoding a viral RdRp, by any procedure useful for the introduction into a particular cell, e.g., physical or biological methods, to yield a transformed cell having the recombinant DNA stably integrated into its genome, so that the DNA molecules, sequences, or segments, of the present invention are expressed by the host cell.

Physical methods to introduce a preselected DNA into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Biological methods to introduce the DNA of interest into a host cell include the use of DNA and RNA viral vectors, e.g., insect virus vectors such as baculovirus vectors. The main advantage of physical methods is that they are not associated with pathological or oncogenic processes of viruses. However, they are less precise, often resulting in multiple copy insertions, random integration, disruption of foreign and endogenous gene sequences, and unpredictable expression.

As used herein, the term "cell line" or "host cell" is intended to refer to well-characterized homogenous, biologically pure populations of cells. These cells may be eukaryotic cells that are neoplastic or which have been "immortalized" *in vitro* by methods known in the art, as well as primary cells, or prokaryotic cells. The cell line or host cell is preferably of mammalian origin, but cell lines or host cells of non-mammalian origin may be employed, including plant, insect, yeast, fungal or bacterial sources.

"Transfected" or "transformed" is used herein to include any host cell or cell line, the genome of which has been altered or augmented by the presence of at least one preselected DNA sequence, which DNA is also referred to in the art of genetic engineering as "heterologous DNA," "recombinant DNA," "exogenous DNA," "genetically engineered," "non-native," or "foreign DNA," wherein said DNA was isolated and introduced into the genome of the host cell or cell line by the process of genetic engineering. The host cells of the present invention are typically produced by transfection with a DNA sequence in a

be synthesized by the solid phase peptide synthetic method. This established and widely used method, including the experimental procedures, is described in the following references: Stewart et al., Solid Phase Peptide Synthesis, W.H. Freeman Co., San Francisco (1969); Merrifield, J. Am. Chem. Soc., 85 2149 (1963); Meienhofer in "Hormonal Proteins and Peptides," ed.; C.H. Li, Vol. 2 (Academic Press, 1973), pp. 48-267; and Bavaay and Merrifield, "The Peptides," eds. E. Gross and F. Meienhofer, Vol. 2 (Academic Press, 1980) pp. 3-285.

When a viral RdRp is expressed in a recombinant cell, it is necessary to purify the polypeptide from other recombinant cell proteins or polypeptides to obtain preparations that are substantially homogenous as to the viral RdRp. For example, the culture medium or lysate can be centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The polypeptide may then be purified from the soluble protein fraction. Alternatively, the polypeptide may be purified from the insoluble fraction, i.e., refractile bodies (see, for example, U.S. Patent No. 4,518,526). The polypeptide may be purified from contaminant soluble or membrane proteins and polypeptides by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; or ligand affinity chromatography, and the like.

If expressed as a fusion polypeptide, the fusion polypeptide may be purified by methods specific for the non-viral RdRp portion of the polypeptide. For example, if the fusion polypeptide is a glutathione-S transferase (GST) fusion polypeptide, GST 4B beads may be employed to purify the fusion polypeptide.

A viral RdRp can also be prepared by *in vitro* transcription and translation reactions. A viral RdRp expression cassette can be employed to generate transcripts which are subsequently translated *in vitro* so as to result in a preparation of substantially homogenous viral RdRp. The construction of vectors for use *in vitro* transcription/translation reactions, as well as the methodologies for such reactions, are well known to the art.

have the arduous task of recognizing many different promoters located both internally and on the termini (Buck, 1996), a situation that was also likely present in the RNA world before the advent of circularized genomes or telomerase functions. Therefore, viral RdRps offer a unique vantage point to gain a better understanding of the progenitor polymerase.

To investigate the mechanism of RNA-directed RNA synthesis, BMV, the type member of the bromovirus group of plant viruses in the alphavirus-like superfamily of (+)-strand RNA viruses, was used. The viral and cellular proteins that comprise the RdRp complex are responsible for directing viral RNA synthesis from the infecting RNA templates, a process which requires specific recognition of salient RNA features. *In vitro*, the BMV subgenomic promoter efficiently and accurately directs (+)-strand RNA synthesis using highly enriched BMV RdRp preparations from infected barley (Miller et al., 1985).

Results

To study BMV subgenomic RNA initiation, RNAs of minimal lengths, designated proscripts as they contain both the promoter (the 20-nucleotides (nts) 3′ of the subgenomic initiation site) and template for (+)-strand RNA synthesis, are employed (Adkins et al., 1997; Siegel et al., 1997). The functional groups at positions -17, -14, -13, and -11 relative to the subgenomic initiation site are required for contact by RdRp during the initiation of RNA synthesis. The sequence-specific recognition of this RNA promoter by a viral polymerase is analogous to the recognition of DNA promoters by DdRp.

To examine BMV RdRp's ability to recognize and accurately initiate RNA synthesis from a DNA version of the subgenomic promoter, DNA templates were used. As the WT control, a 33-nt proscript (designated -20/13) was constructed which contains the WT promoter sequence directing the synthesis of a 13-nt product, the first 11 nts of which are BMV sequence followed by two guanylates which allow labeling of RdRp products with [α-^{32}P] CTP. Standard assays included 25 nM of proscript RNA (unless stated otherwise) with 10 µl of RdRp in a 40 µl reaction containing 20 mM sodium glutamate (pH 8.2), 4 mM $MgCl_2$, 12.5 mM dithiothreitol, 0.5% (v/v) Triton X-100, 2 mM $MnCl_2$, 200 µM ATP and UTP, 500 µM GTP, and 250 nM

three genomic RNAs. To determine whether RdRp recognized the subgenomic initiation site in this fashion (Figure 1), constructs retaining nucleotides at positions -1 to +13 relative to the subgenomic initiation site were synthesized in both RNA and DNA versions, r(-1/13) and d(-1/13) respectively. Removal of the subgenomic promoter from positions -20 to -2, resulting in the placement of the subgenomic initiation site at the penultimate position, did not abolish the ability to direct RNA synthesis (Figure 1, lanes 11 and 15). Furthermore, both the r(-1/13) and d(-1/13) templates were used equally by RdRp (6% vs. 8%, respectively, relative to the amount of synthesis from the -20/13 WT proscript). As observed for proscripts containing the subgenomic promoter, the predominant product was 14 nts for the RNA template, r(-1/13), and 13 nts for the DNA template, d(-1/13).

The requirements for initiation from these templates were determined by mutations surrounding the initiation site. Mutation of the +1 cytidylate or removal of the -1 guanylate abolished RNA synthesis in both r(-1/13) and d(-1/13) templates (Figure 1, lanes 12 and 14; 19 and 21, respectively), demonstrating that initiation must occur at the penultimate position as it does for full-length genomic synthesis. However, RdRp differed in its ability to tolerate the +2 change of an adenylate to a cytidylate in these templates. The +2 a/c mutation abolished the ability to direct RNA synthesis in the r(-1/13) template (Figure 1, lane 13); whereas, RNA synthesis was unaffected by this mutation in the d(-1/13) template (Figure 1, lane 20), indicating a difference in the mode of recognition by RdRp. RNA synthesis from the d(-1/13) template was verified as above; pre-incubation of the DNA template with DNase I abolished RNA synthesis while the product was resistant to DNase I, but degraded by RNase A (Figure 1, lanes 16-18). The RdRp from healthy tomato leaves has also been observed to initiate RNA synthesis from the end of a DNA template, but this initiation did not occur in a sequence-specific manner nor were the requirements for initiation fully characterized (Schiebel et al., 1993).

Hybrid proscripts, containing both ribose and deoxyribose residues, were generated to determine the locations of residues that facilitate RNA synthesis by RdRp (Figure 2). Hybrid H1, only containing riboses in the subgenomic promoter and the +1 and +2 positions, directed an increased amount of RNA

groups, the proscripts were purified and analyzed on anion-exchange HPLC (Wincott et al., 1995). Mass spectral analysis of each chemically synthesized proscript was performed on a Voyager-DE MALDI-TOF spectrometer (Perseptive Biosystem, Framingham, MA) and all were within 0.1% of the expected mass.

Based on electronegativities, all of the substitutions should be capable of forming the ribose sugar conformation at some frequency; however, only the -OH, $-NH_2$, and -F groups are still able to form hydrogen bond contacts while the -OMe group is predicted to be inert (Guschlbauer et al., 1980). The amount of RNA synthesis from the proscripts containing these replacements were determined (Figure 2, lanes 7-11). Each C2′ substitution at position -11 was able to direct RNA synthesis 2- to 3-fold better than the all DNA proscript, d(-20/13), confirming the importance of this functional group. Surprisingly, these substitutions had similar abilities to direct RNA synthesis relative to one another (ranging from 10% to 16%). The level of RNA synthesis from the proscript containing the 2′-OMe substitution (Figure 2, lane 11) clearly shows that the 2′-OH at position -11 is not involved in a hydrogen bond interaction with RdRp. A possible explanation for the importance of this group might be to generate a sugar conformation preferred by RdRp at this position or to prevent some unknown deleterious structure from occurring by steric interference.

A template competition assay was used to evaluate whether the insertion of deoxyriboses in the subgenomic promoter had an adverse effect on the ability to be directly bound by RdRp as would be expected from the functional results (Figure 3). The amount of synthesis from a WT promoter directing the production of a 15-nt product (proscript -20/15) was determined in the absence and presence of various competitor templates. The concentration of the competitor required to reduce the activity from the -20/15 proscript by 50% was termed the I_{50} value. Competitors able to interact more strongly with RdRp will reduce synthesis from -20/15 and have lower I_{50} values.

Proscript -20/13 (composed entirely of ribose residues) reduced the level of 15-nt synthesis by half when present in the same molar ratio as the -20/15 proscript, I_{50} of 25 nM (Figure 3). The ability of d(-20/13) to be bound by RdRp was only mildly affected, having an I_{50} value of 90 nM (Figure 3). This 3- to 4-

of these templates may prevent the need for RdRp to translocate during RNA synthesis. These findings contrast those obtained from other proteins which recognize RNA. As examples, both the MS2 coat protein and *Escherichia coli* alanine-tRNA synthetase directly bind a limited number of 2'-OHs in their cognate RNAs. Since the modern RdRps (or a conserved vestige thereof) best reflect the primitive RNA replicase, these results argue that no significant decrease in binding energy would have occurred during the transition from RNA to DNA templates. The removal of this potential penalty would increase the ease by which an ancestral RdRp could evolve to replicate DNA genomes and gain new functions required in the emerging DNA world.

Example 2

Genomic RNA synthesis by BMV RdRp

Materials and Methods

Synthesis of DNA and RNA templates for RdRp assay

Synthetic deoxyoligonucleotides used as template for RdRp assays were purchased from Operon Technology. All oligonucleotides were quantified by spectrophotometry, adjusted to the concentration desired for manipulation, and visually inspected after staining with Toluidine blue after gel electrophoresis. RNA templates, were made by PCR followed by *in vitro* transcription. First, PCR was used to synthesize the cDNA of choice using two oligonucleotides, one of which contained a T7 promoter. Thirty cycles of PCR was carried out with the appropriate template using Taq polymerase. Each cycle consisted of 30 sec of denaturation at 94° C, annealing at 5° C below the lowest oligonucleotide T_m, and elongation at 72° C. PCR products were purified using standard protocols (Sambrook et al., 1989) followed by *in vitro* transcription (Ampliscribe, Epicentre). Transcripts were purified by anion exchange chromatography on Qiagen tip-20 columns using the manufacturer's protocol. Concentration of RdRp templates was determined by Toluidine blue staining following denaturing PAGE and by spectrophotometry.

RdRp activity assays

BMV RdRp was prepared from infected barley as described by Sun et al. (1999). Standard RdRp activity assays consisted of 40 µl reactions containing 20 mM sodium glutamate, pH 8.2, 4 mM $MgCl_2$, 12 mM dithiothreitol, 0.5%

The requirements for the initiation of RNA synthesis from the penultimate nucleotide were examined first. The removal of the 3'most nucleotide reduced RNA synthesis to 10% of d(-1/13) (Figure 5A, lane 3). However, the 3'-most guanylate can be replaced with a uridylate, cytidylate, or an adenylate and direct synthesis between 38 to 63% of d(-1/13). Addition of a cytidylate at the 3'-most position should place a potential initiation nucleotide at both the 3'-end terminus and the penultimate position. However, initiation still took place from the penultimate cytidylate as judged by the mobility of the resultant RNA. The addition of more than one nucleotide (AG) 3' of the initiation cytidylate decreased the amount of synthesis to 33%. However, the resulting products were indistinguishable in size from those produced by d(–1/13) wild type, suggesting that initiation still took place from the authentic cytidylate. Addition of three nucleotides (AAG) 3' of the initiation cytidylate resulted in synthesis at 14 % of that from d(-1/13). These results indicate that the penultimate nucleotide is the preferred nucleotide and that additional sequence 3' of the initiation nucleotide can affect the efficiency of RNA synthesis.

Replacement of nucleotides at position +2 to +7

The sequence immediately 5' of the initiation site is rich in A-U nucleotides. All possible changes of nucleotides from position +2 to +4 were made to examine the requirements for RNA synthesis (Figure 5A, lanes 8-16). Replacement of the +2 adenylate with uridylate resulted in synthesis similar to wt d(–1/13). However, changing the +2 adenylate to a guanylate or a cytidylate reduced synthesis to less than 30% of wt d(–1/13). At the +3 position, transition from a uridylate to a cytidylate was acceptable for RNA synthesis while transversion to purines decreased RNA synthesis to less than 20% of wt d(–1/13). At the +4 position, changing adenylate to either an uridylate or a cytidylate resulted in wt d(–1/13) levels of RNA synthesis while a change to a guanylate decreased RNA synthesis to 21% of wt d(–1/13).

The presence of a guanylate at positions from +1 to +4 was consistently detrimental to efficient RNA synthesis (Figure 5D). To examine this correlation further, positions +5 and +6, normally uridylates, were individually changed to guanylates. A change of +5 U/G yielded same amount of synthesis as d(-1/13),

In contrast to the importance of the –1 and +1 positions, changes in later positions along the template had decreased effects on stable interaction with RdRp. Although the change of +2 A/G had an adverse effect on its ability to direct RNA synthesis, all the nucleotide combinations at the +2 position tested resulted in inhibition of r(–20/15) synthesis at levels similar to wt d(–1/13). At position +3 and +4, changes of the original template nucleotides to guanylates had adverse effects on synthesis, but not on the ability of the templates to interact with RdRp (Figure 6). Changes at the +5 and +6 positions resulted in interaction with RdRp at levels comparable to wt d(–1/13) (Figure 6).

A more quantitative analysis of how specific DNAs affect the ability of RdRp to interact with r(–20/15) was tested. DNA competitor was added in molar excess to decrease synthesis of r(–20/15) below 50%. The concentration of the competitor DNA required to reduce synthesis by 50% was termed I_{50}. Lower values indicate that the competitor can interact more stably with RdRp and prevent synthesis from r(–20/15) at a lower concentration. The I_{50} value for wt d(–1/13) was 170 nM. An oligonucleotide which contains deoxythymidines instead of deoxyuridine had a similar I_{50} value of 150 nM (Figure 7). These results show that the C5 methyl group which distinguishes deoxythymines from deoxyuracils do not have an obvious effect on the ability of the DNA template to interact with RdRp. Consistent with the inhibition assay results shown in Figure 6, removal of the 3' terminal guanylate or changing the initiation cytidylate to a guanylate raised the I_{50} values to approximately 1 mM. These results indicate that the primary requirement for RdRp-DNA template interaction is with the –1 and +1 nts.

Synthesis of genomic positive strand RNA from minus strand "endscripts"

The initiation of genomic positive strand RNA synthesis from minus strand templates was not previously observed with BMV RdRp. The results from the DNA template studies described above suggest that an additional nucleotide 3' of the initiation cytidylate may be required. This may be expected since the presence of this non-template nucleotide in the minus strand RNA would not direct the incorporation of an extra nt. Siegel et al. (1997) have demonstrated that BMV RdRp could add a non-templated nucleotide at the 3' end of an RNA molecule by a terminal transferase activity.

B2(-) endscripts 200-nt in length were generated by *in vitro* transcription. B2(-)200G, in addition, had an extra guanylate at its 3' end. After a standard RdRp reaction, the products were analyzed on a 5% denaturing PAGE. B2(-)200G was able to direct efficient synthesis, whereas reduced amount of synthesis (30%) was observed in the absence of a 3' guanylate (Figure 8D). The amount of synthesis observed in the absence of a 3' non-templated nucleotide may be due to addition of extra nucleotide(s) by the T7 polymerase during transcription (Cazenave et al., 1994; Pleiss et al., 1998). To confirm that initiation of the 200-nucleotide product took place from the penultimate cytidylate, the +1 cytidylate was mutated to a guanylate. As expected endscripts from this mutant construct was unable to direct positive strand synthesis (Figure 8D). In addition to the 200-nucleotide product, a 100-nucleotide product was also observed. This appears to be due to internal initiation of synthesis, since mutation of the +1 cytidylate did not affect its synthesis (Figure 8D). As shorter endscripts are more conducive to thorough analysis of template requirements, all experiments described below use the B2(-)46G as prototype.

Effect of nucleotide changes at endscript positions near the initiation cytidylate

In addition to the nucleotide at the –1 position, the effect of nucleotide changes at +1, 2 and 3 positions on RNA synthesis was determined. Consistent with previous observations, changing the +1 cytidylate to a guanylate was detrimental and virtually abolished RNA synthesis (Figure 9A, lane 3). Changing the +2 adynylate to a guanylate was also detrimental, reducing synthesis to 8% of wildtype (Figure 9A lane 4)). These results are comparable to observations made using DNA templates containing similar changes at the +1 and +2 positions (Figure 5C). However, changing the +3 uridylate to an adenylate did not affect RNA synthesis from an RNA template, in contrast to RNA synthesis from a DNA template with the identical nt change (compare Figure 5C and 9A lanes 1, 5). The lack of a 2' OH and/or the presence of 5' methyl group at specific positions may slightly alter the interaction between the template and RdRp.

Effect of minus strand 3' end on RNA synthesis

To further analyze the effect of 3' non-templated nucleotide on RNA synthesis, endscripts were generated containing differing nucleotides at the 3'-

CMV RNA has identical sequences as BMV at the 3' end (Figure 8A). Taken together these results clearly indicate that nts at positions –1, +1, and +2 are important for the binding of the RdRp complex.

To determine if RdRp could discriminate between multiple initiation sites varied by position within a template and by different spatial relationship to 5' sequences, endscripts containing one, two or three initiation sites ($C_{+1}A_{+2}U_{+3}$) were assayed in a standard reaction. With the endscript containing two initiation sites, the products observed were 46- and 49-nucleotides corresponding to initiation from the authentic and the 3'-most initiation sites, respectively. The 49-nucleotide product initiating from the penultimate cytidylate was 89%, while the 46-nucleotide product was at 25% in comparison to the wt B2(-)46G (Figure 10). When three initiation sites were present, products of 52-, 49-, and 46-nucleotides corresponding to initiation from all three potential sites were observed at 69%, 43%, and 19% respectively. Since the 3' terminal initiation site always yield the most product, this result confirms previous observations that RdRp has a preference for the penultimate cytidylate (Figure 10). However, it is also interesting to note that as the 3' end is extended, the authentic initiation site is still used in endscripts with two or three initiation sites. The use of the authentic initiation site suggests that an appropriate spacing between the 3' initiation site and sequences or structure requiring the sequences 5' of the initiation site may influence the efficiency of positive strand RNA synthesis.

5' requirements to initiate positive strand synthesis

The B2(-)46G endscript is predicted to fold into a stable stem-loop structure that was named L1, A1, and A2 (Figure 11A). In order to determine whether the RNA structure or some sequences present 5' of the initiation site was important for directing positive strand synthesis, a set of deletions were constructed and tested. Deletion of sequences 3-11 is expected to disrupt the A2 stem, and deletion of sequences 17-26 is expected to disrupt the L1 loop region. When sequences 3-11 were deleted, disrupting the A2 stem, synthesis (after adjusting to the number of radiolabeled CMP incorporated) was not adversely affected (Figure 11B lanes 5-6). In fact deleting the predicted stem A2 reproducibly increased synthesis to 200% as compared to wt B2(-)46G (compare Figure 11B lanes 1-2 and 5-6). Deletion of nts 17-26, disrupting the L1 loop region, resulted in an RNA that directed 70% of wt RNA synthesis (Figure 11B lanes 9-10). In both cases, initiation took place from the authentic +1 cytidylate, as a change to a guanylate resulted in RNAs that failed to direct synthesis

Since the 5' end affects initiation of RNA synthesis, it was determined whether the sequence from nucleotides 17-26 is sufficient for interaction with RdRp. To address this question, B2(-) sequence 11-26 (R11-26) and the transversion of this sequence at positions 17-26 (TV11-26) were chemically synthesized for use in template competition assays. Addition of either R11-26 or TV11-26 to ten-fold molar excess of the reference RNA r(-20/15), did not reduce RNA synthesis. As controls, assays carried out in parallel with competitor B2(-)26G or B2(-)26TV were able to efficiently compete. These results indicate that nucleotides in the 11-26 region in the absence of the 3' sequence are not sufficient for stable interaction with RdRp.

Discussion

The results described above demonstrate for the first time accurate *in vitro* initiation of genomic positive strand RNA synthesis by BMV RdRp. Using DNA templates that mimicked the 3' end of BMV RNA minus strand, it was found that recognition of minus strand templates by RdRp *in vitro* required a non-templated nucleotide. Addition of this non-templated nucleotide to the 3' ends of the minus strand of all three BMV RNAs allowed accurate initiation of genomic positive strand RNAs in a species-specific manner. RdRp recognition of minus strand template requires nucleotides present at the 3' initiation site but synthesis is affected by 5' sequences.

Comparison of synthesis from RNA and DNA templates

RNA synthesis directed by DNA templates is about 8% as efficient as using RNA templates. However, the requirements for template recognition by RdRp appears to be very similar in both DNA and RNA. The −1, +1 and +2 positions in a DNA sequence of BMV template were important for recognition by RdRp. The results from the +3 position suggests that a U/A change is tolerated in an RNA template. Whereas, in a DNA template, the change of a 2' OH to a 2' H and/or a change of a U to a T, which has an additional C5 methyl group, may induce perturbations and could explain the inability of RdRp to effectively recognize the template. The presence of a guanylate at the +1 to +4 positions was also found to be detrimental for directing efficient synthesis. Use of DNA templates for i*n vitro* studies had advantage in that generation of DNA templates with precise ends is possible by chemical synthesis, whereas, RNA

these transcripts generated by T7 RNA polymerase could have an extra nucleotide at their 3' ends as discussed previously, and may help explain why higher levels of synthesis was observed with the B2(-)46 RNA template lacking an extra 3' nucleotide (Figure 9A).

The 3' end of BMV and CMV RNAs have identical sequences (Figure 8A). However, the CMV RNAs are poor templates for RNA synthesis, indicating that additional sequences 5' of the initiation site are important for efficient synthesis, suggesting that 5' sequences confer specificity in viral RNA synthesis. The 5' sequences may affect synthesis by three possible mechanisms: 1) affect abortive synthesis, 2) allow antitermination of RNA synthesis (Burns et al., 1998: Richardson, 1996) or 3) affect initiation at the 3' end (but not RdRp binding). It is possible that BMV RdRp will abort synthesis when utilizing non-BMV templates. However, since abortive synthesis usually terminates before the first ten phosphodiester bonds are formed (Sun and Kao, 1997a; Sun and Kao, 1997b), the BMV sequence between 17 and 26 may not be directly involved in abortive synthesis but could be inducing elongation or affecting initiation. Further analysis of the 5' sequences is required to determine its precise role in directing positive strand synthesis.

Pogue and Hall (1992), have suggested that the putative positive strand stem-loop structure and the ICR2-like sequences present within the loop region are important for genomic positive strand synthesis. When they disrupted a putative positive strand stem-loop structure, RNA replication was greatly reduced. Whereas, disruption of the putative minus strand stem-loop structure was not correlated with effects on replication (Pogue and Hall, 1992. These results led them to propose that the positive strand RNA is involved in additional rounds of positive strand RNA synthesis. As demonstrated above, accurate initiation of positive strand RNA synthesis can take place from minus strand templates in the absence of positive strand RNAs (and the ICR-like sequences). In addition, we have demonstrated that disrupting the predicted A2 stem structure does not affect the template's ability to direct RNA synthesis. Contrary to the conclusions of Pogue and Hall (1992), specificity signals required for binding and processive RNA synthesis to be present on the minus strand template. While the results described herein do not rule out possible

Triton X-100, 1 mM $MnCl_2$, 200 μM ATP and UTP, 500 μM GTP, and 250 nM α-[P^{32}]-CTP (Amersham). Manganese is used to increase the level of RNA synthesis. *de novo* initiation does occur from (-)21g in the absence of Mn2+. Reactions were incubated at 25°C for 60 minutes and stopped by phenol/chloroform extraction followed by ethanol precipitation in the presence of 5 μg of glycogen and 0.4 M ammonium acetate. Products were separated by electrophoresis on 20% denaturing (8 M urea) polyacrylamide gels. Gels were wrapped in plastic and exposed to film at -80°C. Product bands were quantified using a Phosphorimager (Molecular Dynamics), and values were compared to the amount of product generated from the wild-type template (-)21g to derive the relative percent activity of mutant templates. All values represent the mean of at least three independent experiments.

Results

De novo initiation of RNA synthesis. To examine the mechanism of RNA synthesis by the recombinant BVDV NS5B protein, NS5B tagged with six histidines at its C-terminus was expressed using a baculovirus vector and purified by Ni-NTA affinity chromatography as described in Zhong et al. (1998). Preparations of purified NS5B contained a predominant protein of the expected size of 75 kDa (Figure 5A).

The first template for polymerase reaction was a chemically synthesized RNA named (-)21g that corresponded to the 21 nucleotides at the 3′ end of the (-)-strand BVDV genome. This template was used because previous work on brome mosaic virus genomic (+)-strand initiation revealed fewer template requirements than genomic (-)-strand synthesis (Example 2). A guanylate was added to the 3′ end of the BVDV 21-nt (-)-strand sequence for two reasons: 1) NS5B has been reported to have terminal transferase activity (Zhong et al., 1998), which may add one or more nucleotides to the end of the RNA *in vivo*; 2) the brome mosaic virus RdRp must initiate RNA synthesis from the penultimate nucleotide and the lack of a nontemplated nucleotide prevents synthesis (Example 1). To more easily discern *de novo* initiation from primer-extension, the 3′ terminal guanylate in (-)21g was modified to have a dideoxyribose (Figure 13B). The lack of 2′ and 3′ hydroxyls rendered the template incapable of priming RNA synthesis.

concentrations of GTP for the initiation nucleotide by the BMV RdRp (Kao et al., 1996) and that lower concentrations of GTP are sufficient for the steps after the initiation of RNA synthesis (Lohmann et al., 1998; Kao et al., 1996). To determine whether this is also true for the BVDV NS5B, the NTP mix in the reaction was adjusted to contain only 2 μM GTP while the concentrations of other nucleotides, including the α-^{32}P-CTP, were unchanged from previous reactions. This lower level of GTP did not allow observable product synthesis (Figure 13E, lanes 1 and 2). Consistent with this, attempts to use γ-[^{32}P]-GTP as the sole GTP in a radiolabeling experiment (present at 0.3 μM in these reactions) also resulted in no observable product synthesis. Reactions containing 2 μM GTP were then supplemented with 500 μM final concentration of either GMP or GDP. RNAs of 21- and 22-nts were then easily observed (compare lanes 1-4). GMP also resulted in a smaller molecular weight product of less than 14-nts. Taken together, these results suggest that BVDV NS5B requires higher concentrations of guanylate for initiation than for elongation.

De novo initiation from DNA templates. RdRp has been reported to initiate RNA synthesis from templates containing deoxynucleotides (Siegel et al., 1998). DNA d(-)21g was synthesized to use in reactions with the BVDV NS5B protein (Figure 6A). In addition to the change of the 2′ OH to a 2′ deoxy, d(-)21g contains thymines in place of uridines, adding a C5-methyl group to the uridines. DNA d(-)21g was able to direct the synthesis of the 21- and 22-nt products (Figure 6B, lanes 3 and 4). A change of the +1 cytidylate to a guanylate in the DNA abolished synthesis (lanes 5 and 6), indicating that initiation from d(-)21g takes place from the penultimate cytidylate. Furthermore, the amount of RNA synthesized from d(-21g) was similar to that from (-)21g, indicating that the lack of 2′OH in the ribose and the addition of C5 methyl groups to uridines did not impair NS5B-template interaction.

One difference between synthesis from DNA and RNA templates is that d(-)21g produced slightly higher levels of truncated products of between 11- to 20-nts. These products are initiated correctly as judged by the lack of these products in a template in which the +1 cytidylate was changed to a guanylate (compare Figure 14, lanes 4 and 5). The synthesis from a single-stranded DNA template is thus similar overall, but not identical to that from an RNA template.

In order to distinguish the initiation nucleotide(s) used for these products, RNA 3'gGCC was tested, which changed the +1 cytidylate to a guanylate but retained cytidylates at the +2 and +3 positions (Figure 14A). This RNA directed decreased amount of synthesis of all RNA products 21-nts and longer (Figure 14B, lane 8), indicating that all of the higher molecular weight products are the result of initiation from the +1 cytidylate. Due to its slight decrease in size in comparison to the 21-nt RNA, the lone remaining product of 20-nts is likely due to initiation of RNA synthesis from the +2 cytidylate. The putative 20-nt product may be made only when NS5B is unable to use the preferred initiation positions.

To confirm the preference for initiation from the 3' end of the RNA template, RNAs containing two or three consecutive copies of nucleotides +1 to +3 (3' CAU 5') were tested. These templates, named 2-init and 3-init (Figure 14A) could potentially initiate synthesis at multiple positions. As judged by the sizes of the RNAs synthesized, the predominant initiation cytidylate used in both 2-init and 3-init was the cytidylate proximal to the 3' terminus (Figure 14B, lanes 10 and 11). These results also indicate that there is no detectable spatial requirement between the initiation site and sequences 5' of the initiation site. These data, taken collectively, suggest that the recognition of the initiation site requires a cytidylate present at or near the 3' end of the template.

Interaction between NS5B and the initiation site. A template competition assay was used to evaluate whether nucleotide changes from (-)21g affect the ability to interact with NS5B. The amount of synthesis from 3-init directing the production of a 27-nt RNA in the absence and presence of various competitor RNAs was determined. All of the experiments were performed using limiting amounts of NS5B. Competitors that are able to interact more strongly with RdRp will better reduce synthesis from 3-init. The competitors used were chosen primarily because they are crippled in the ability to direct RNA synthesis. This feature reduces the possibility that a competitor is reducing synthesis due to competition for limited amounts of NTP in the polymerization reaction. However, it is unlikely that NTPs are the limiting factors in these reactions since the addition of more polymerase increases the amount of synthesis.

is the only known class of template-dependent polymerases that can initiate RNA synthesis *de novo* from the terminus of the template. Telomerase, which directs DNA synthesis from the end of chromosomes, uses an endogenous RNA to guide polymerization (Ligner et al., 1995). Chemically synthesized RNAs were used to examine the requirements for the initiation of genomic (+)-strand RNA synthesis by recombinant NS5B. A cytidylate positioned at or near the 3′ end of the RNA or DNA was able to direct RNA synthesis. In the initiation of RNA synthesis, GTP, the first nucleotide in the nascent RNA, is needed in higher concentrations. This GTP can be replaced with GDP and GMP. In addition, a pyrimidine in the +1 position of the template contributes to the stable interaction with NS5B, perhaps through basepairing with a GTP (Siegel et al., 1998). Interestingly, only a +1 cytidylate can efficiently direct RNA synthesis while a +1 uridylate is acceptable for NS5B interaction. A model summarizing the results for NS5B-RNA interaction is presented in Figure 16C.

The results described herein are in contrast to the previous observations of Lohmann et al. (1997; 1998) and DeFrancesco and colleagues (1996). As described above, predominantly *de novo* initiation of RNA synthesis from either RNA or DNA templates was observed while others have observed only RNA synthesis by extension from the 3′ end of the template or from an oligonucleotide (hereafter defined as elongative synthesis). Indeed, previous work using BVDV (Zhong et al., 1998 and unpublished data) demonstrated that the BVDV NS5B protein also can perform elongative RNA synthesis with certain templates.

One or more of several possibilities may account for the different observations. First, all the previous work used template RNAs that were significantly longer than those described above. Perhaps in a longer RNA, secondary structures will either induce elongation by the RdRp or inhibit *de novo* initiation. Alternatively, it is possible that RNA sequences needed to induce the initiation of RNA synthesis *in vivo* may be kept more accessible due to the binding of viral proteins. The oligomerization of the poliovirus RdRp on the template RNA, a process that would affect RNA structure, has been previously observed (Pata et al., 1995). Second, a relatively strict positional preference for the initiation nt was observed (Figure 14). The previously described RNAs may

Establishing the conditions for *de novo* RNA synthesis by a recombinant RdRp facilitates the determination of the requirements for the initiation of RNA synthesis. These results may also permit identification of the amino acid residues in NS5B that are responsible for activities such as binding of the initiation GTP, the second nucleotide, and the template RNA. In addition, this system may be useful for a mechanistic study of RNA recombination. In several of the reactions, higher molecular weight products were observed (for example, Figure 5C, lanes 5 and 6). These products are likely due to NS5B using the nascent RNA as a template for additional rounds of synthesis. If true, this represents a template-switch event that is the basis for RNA recombination (reviewed in Lai, 1992; and Nagy et al., 1997). Indeed, many naturally occurring isolates of BVDV contain genomic sequence deletions or insertions derived from either viral RNA or host mRNAs. These are all believed to arise from RNA recombination events (for examples, see Tautz et al., 1994; Tautz et al., 1996; and Meyer et al., 1996).

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

7. The method of claim 1 or 2 wherein the viral polymerase is that of a positive strand RNA virus.

8. The method of claim 1 or 2 wherein the viral polymerase is hepatitis C virus polymerase.

9. The method of claim 1 or 2 wherein the template comprises signals for the initiation of genomic (+) strand synthesis.

10. An agent identified by the method of claim 2.

11. A diagnostic method, comprising:

(a) contacting a physiological sample from a mammal infected with, or suspected of being infected with, a positive strand RNA virus with recombinant viral RNA-dependent RNA polymerase under conditions that result in *de novo* initiation of RNA synthesis so as to yield a RNA product; and

(b) detecting or determining the presence or amount of the RNA product relative to the presence or amount of control RNA product yielded from contacting the physiological sample from a mammal that is not infected with a positive strand RNA virus under conditions that result in *de novo* initiation of RNA synthesis.

12. A method to inhibit or reduce viral infection or replication, comprising administering to a mammal infected with, or suspected of being infected with, a positive strand RNA virus, the genome of which encodes a viral RNA-dependent RNA polymerase, an amount of an agent effective to inhibit *de novo* initiation of RNA synthesis by the viral RNA-dependent RNA polymerase.

13. A method to detect viral-specific nucleic acids, comprising:

(a) contacting a sample suspected of having a viral-specific nucleic acid molecule with an isolated viral RNA-dependent RNA polymerase under conditions that result in *de novo* initiation of RNA synthesis so as to yield a RNA product; and

25. The kit of claim 22 wherein the modified nucleotide has a modified phosphate group.

26. The kit of claim 22 wherein the modified nucleotide is detectable.

27. The kit of claim 26 wherein the modified nucleotide is chemically altered.

28. The kit of claim 26 wherein the modified nucleotide is fluorescently labeled.

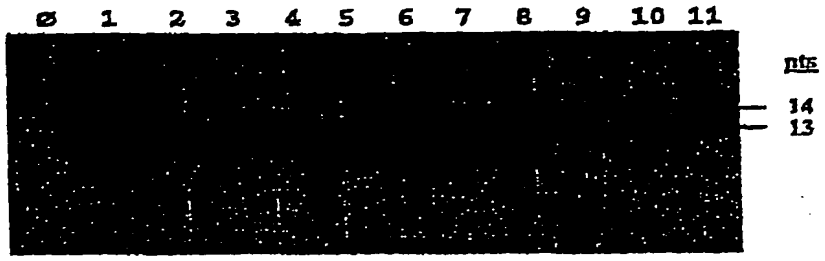
29. The method of claim 1 or 2 wherein the template comprises deoxyribonucleotides and ribonucleotides.

30. The method of claim 1 or 2 wherein the template comprises a modified nucleotide.

A.

Proscript Name			Lane
-20/13 WT	3′	CUAGAUACAGGAUUAAGUCGCAUAAUUAUUAGG 5′	1
H1		CUAGAUACAGGAUUAAGUCGCAuaauuauuagg	2
H2		CUAGAUACAGGAUUAAGUCGcauaauuauuagg	3
H3		cuaGauACaGgauuaagucgCAuaauuauuagg	4
H4		CUAGAUACAGGAUUAAGUCGCAtaattattagg	5
-17 g/2′-H		CUAgAUACAGGAUUAAGUCGCAUAAUUAUUAGG	6
-11 g/2′-H		CUAGAUACAgGAUUAAGUCGCAUAAUUAUUAGG	7
d(-20/13)		cuagauacaGgauuaagucgcauaauuauuagg	
-11 g/		2′-OH	8
		2′-NH_2	9
		2′-F	10
		2′-OCH_3	11

B.



	ø	1	2	3	4	5	6	7	8	9	10	11
% Activity	0	100	20	19	32	17	145	46	13	10	14	16
SD +/-			6	5	1	4	15	5	1	1	2	2

Figure 2

A

			I_{50} (μM)
RNA -20/15 WT	3' CUAGAUACAGGAUUAAGUCGCAUAAUUAUUAUUGG 5'		
DNA Inhibitors	d(-1/13)	3' d(gcauaauuaucagg) 5'	0.350
	d(-1/13) Rev	d(ggactattaatacg)	>> 12.5
	d(-1/8)	d(gcauaaugg)	4.5
	d(-1/6)	d(gcauagg)	9.5

B

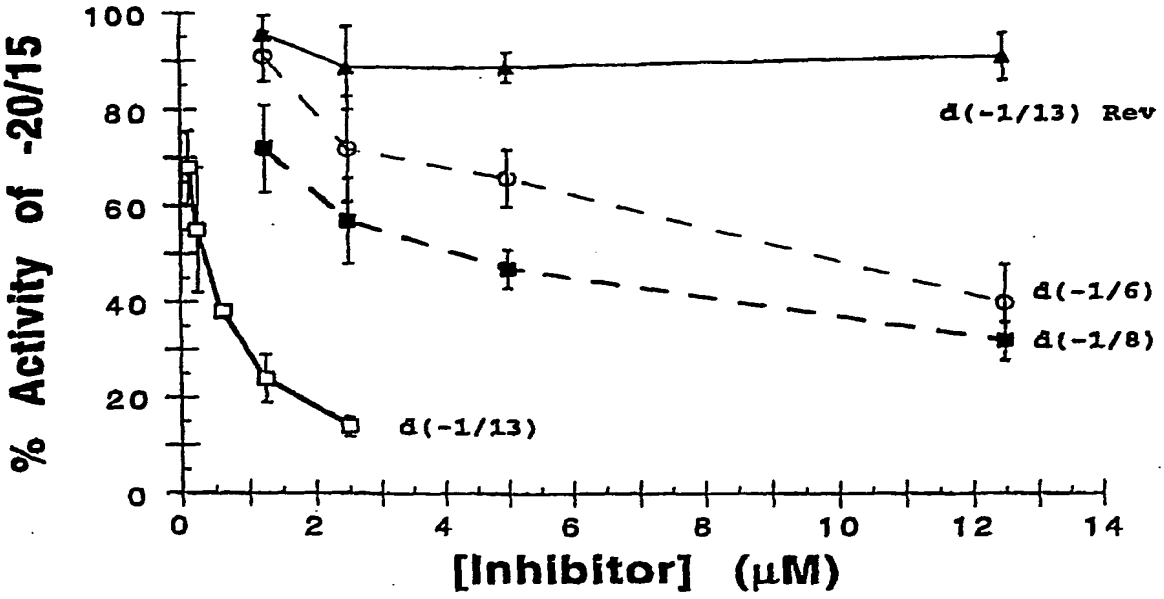


Figure 4

Name	Sequence	%synthesis	% Inhibition of r(-20/15) 5X	10X
Wt	3' GCAUAUUAAUACC	100	60 +/- 12	43 +/- 6
+1/13	Δ----------	10	95+/- 10	90 +/- 2
3'C	C----------	38	75 +/- 10	ND
+GAA	AA----------	14	94 +/- 6	ND
+1C/G	--G----------	5	98 +/- 13	62 +/- .3
+2G	---G----------	29	64 +/- 12	38 +/- 1
+2U	---U----------	118	73 +/- 9	46 +/- 5
+3G	----G----------	7	82 +/- 3	58 +/- 3
+3A	----A----------	19	ND	47 +/- 1
+4G	---------G----------	21	79 +/- 5	56 +/- 5
+5G	----------G----------	110	57 +/- 9	47 +/- 3
+6G	----------G----------	40	60 +/- 2	43 +/- 4

Fig. 6

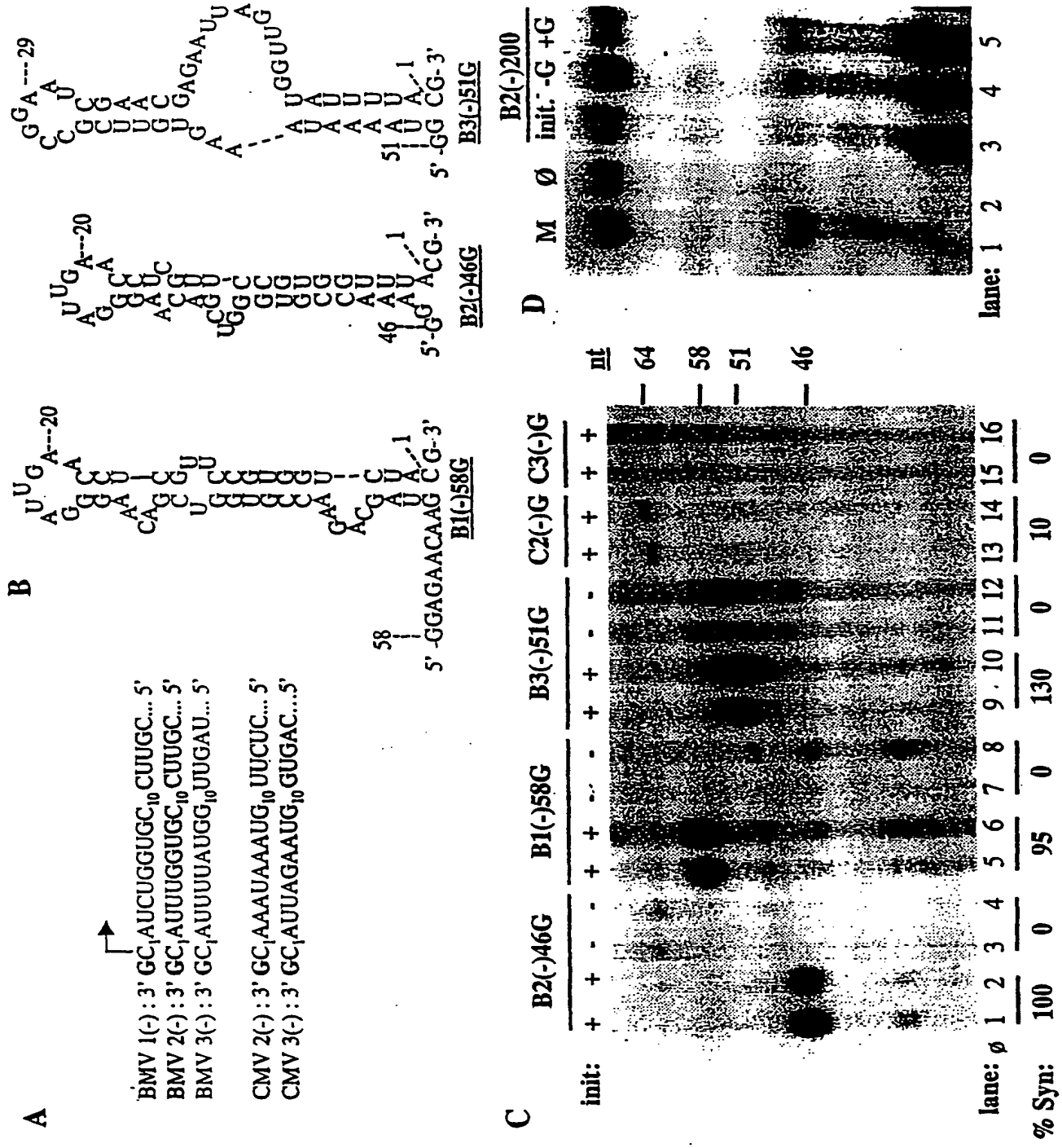


Fig.8

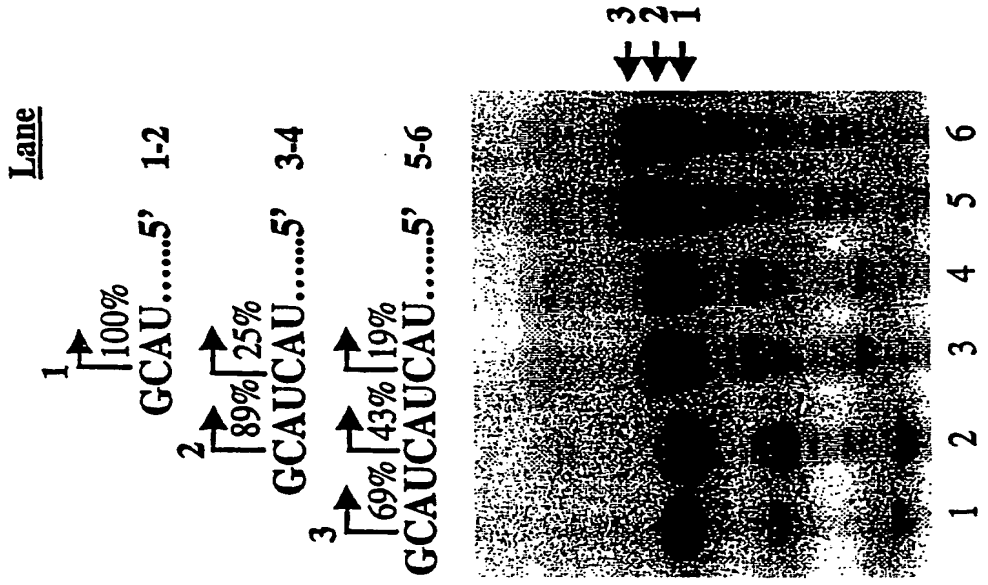


Fig. 10

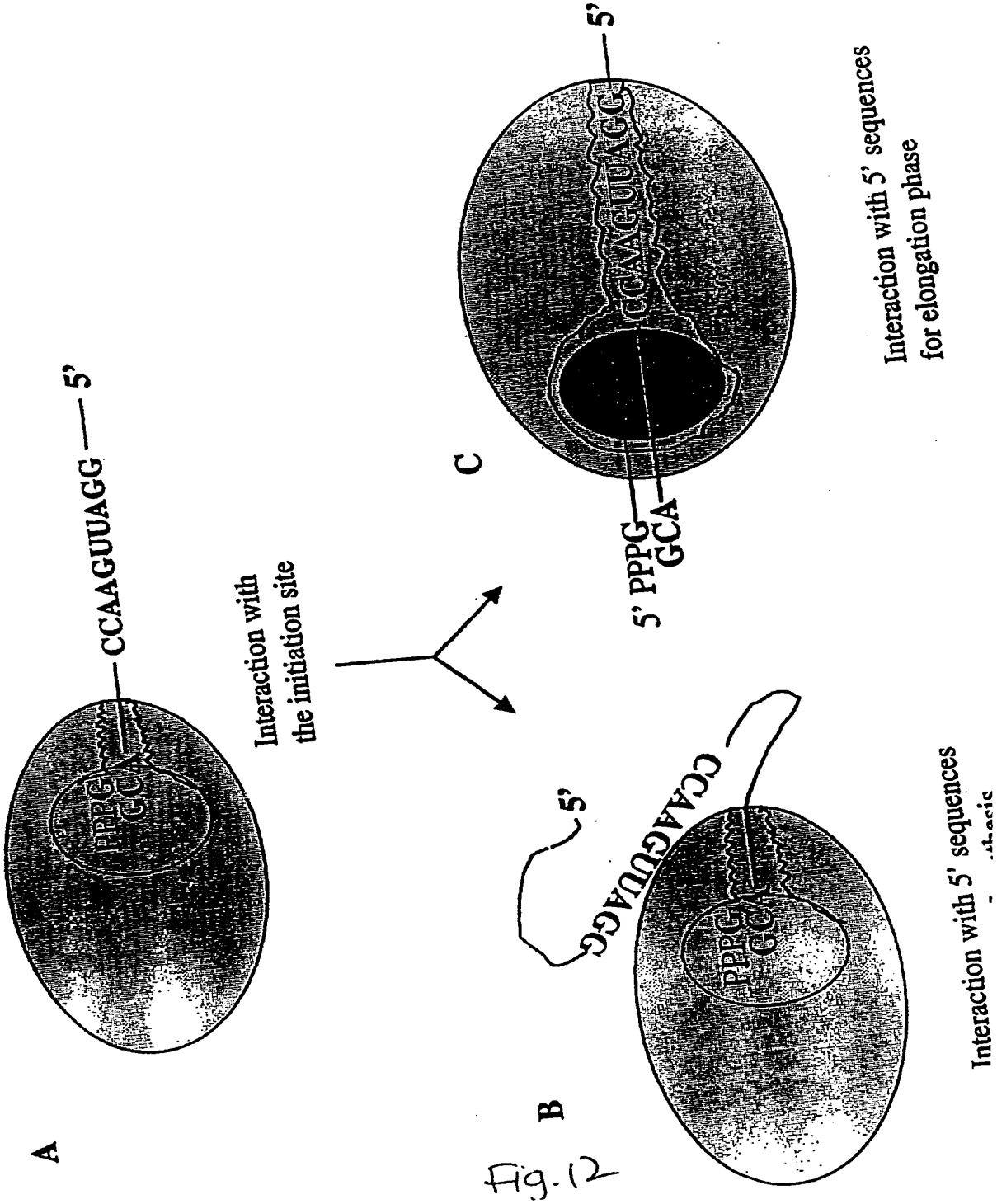


Fig. 12

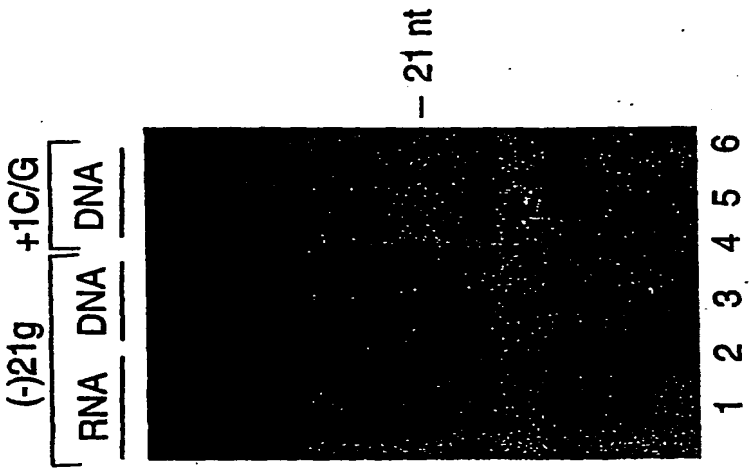
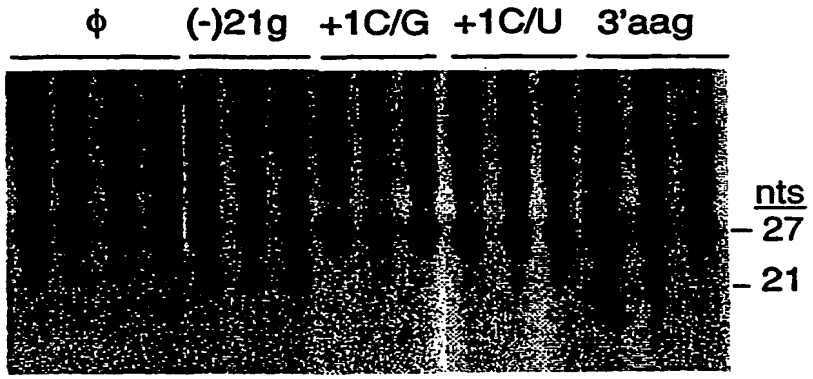


Figure 14

A



B

RNA Comp.:	5 pm	20 pm
None (100%)		
Wt (-)22	35.7+/- 6	23.8 +/- 5.7
+1C/G	83.9+/- 11.2	44.8 +/- 7.5
+1C/U	47.9 +/- 11	26.6 +/- 4.3
3'ag	57.7 +/- 12.7	29.9 +/- 2
3' aag	72 +/- 4	44.8 +/- 6

C

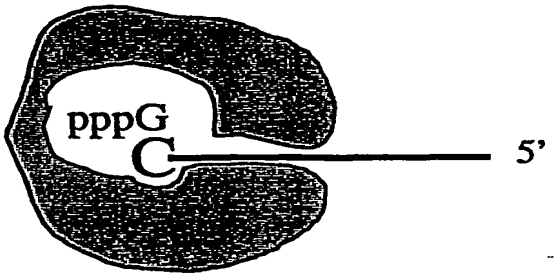


Figure 16

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/70, G01N 33/50 — A3

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(71) Applicant: ADVANCED RESEARCH AND TECHNOLOGY INSTITUTE, INC. [US/US]; Suite 100, 1100 Waterway Boulevard, Indianapolis, IN 46202 (US).

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(74) Agent: VIKSNINS, Ann, S.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(57) Abstract

An isolated recombinant viral polymerase is provided that is useful in diagnostic and anti–viral compound screening applications.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/00152

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/70 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LOHMANN V ET AL: "BIOCHEMICAL PROPERTIES OF HEPATITIS C VIRUS NS5B RNA-DEPENDENT RNA POLYMERASE AND IDENTIFICATION OF AMINO ACID SEQUENCE MOTIFS ESSENTIAL FOR ENZYMATIC ACTIVITY" JOURNAL OF VIROLOGY,US,THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 71, no. 11, November 1997 (1997-11), pages 8416-8428, XP000877461 ISSN: 0022-538X the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search	Date of mailing of the international search report
3 July 2000	20. 07. 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/00152

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 12,14-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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WO 00/40759 A3

(54) Title: USES OF FLAVIVIRUS RNA-DEPENDENT RNA POLYMERASES

(57) Abstract: An isolated recombinant viral polymerase is provided that is useful in diagnostic and anti-viral compound screening applications.

DNA replication revealed that the initiation of each of these processes is the most highly regulated. Thus, the initiation of viral RNA replication is likely to be the most regulated step in viral RNA replication. The initiation of viral RNA synthesis takes place at the very end of viral nucleic acids, which is distinct from the initiation of cellular nucleic acid synthesis. This difference makes viral initiation a very attractive target for the development of antivirals.

Positive strand RNA viruses in the *Flaviviridae* family include human and animal pathogens such as flavivirus, hepatitis C virus (HCV), and the pestivirus bovine viral diarrhea virus (BVDV) (Thiel et al., 1996). After entry, the viral RNA directs the translation of a polyprotein that is proteolytically processed to produce individual structural and nonstructural proteins (Collett et al., 1988; Xu et al., 1997). At the C-terminus of the polyprotein is the nonstructural protein 5B (NS5B), an RdRp that is a key subunit of the viral RNA replicase complex. Recombinant NS5B of HCV and BVDV has been previously reported to be able to catalyze nucleotidyl transfer by extending from the 3′ hydroxyl of a template, an RNA or DNA primer (DeFrancesco et al., 1996; Lohman et al., 1997; Lohmann et al., 1998). However, the ability of recombinant NS5B to initiate RNA synthesis by a primer-independent mechanism, a mechanism that likely occurs in the infected cell, has not been previously reported.

Thus, what is needed is a method to determine whether a RdRp can initiate RNA synthesis *de novo*.

Summary of the Invention

The invention provides an isolated viral RNA-dependent RNA polymerase (RdRp) that can initiate nucleic acid synthesis *de novo* from either a RNA or DNA template, and methods which employ such a polymerase. Preferably, the RdRp is a recombinant protein. Preferably, the template comprises signals for the initiation of genomic (+) RNA strand synthesis, and is a linear molecule, e.g., at least a portion of the linear genome of a viral pathogen.

Polymerases within the scope of the invention are preferably polymerases from viruses having single strand (+)-sense RNA genomes, and more preferably polymerases from the Flaviviridae family, e.g., flaviviruses, pestiviruses and Hepatitis C virus (see Fields, Virology, 3rd. Edition Schlesinger

(b) detecting or determining the presence or amount of the RNA product in the presence of the agent relative to the presence or amount of control RNA product yielded from contacting a mixture comprising an isolated recombinant viral RNA-dependent RNA polymerase and a nucleic acid template under conditions that result in *de novo* initiation of RNA synthesis, wherein a reduced amount of the RNA product in the presence of the agent is indicative that the agent inhibits *de novo* initiation of RNA synthesis.

Also provided is a diagnostic method. The method comprises:

(a) contacting a physiological sample from a mammal infected with, or suspected of being infected with, a positive strand RNA virus with recombinant viral RNA-dependent RNA polymerase under conditions that result in *de novo* initiation of RNA synthesis so as to yield a RNA product; and

(b) detecting or determining the presence or amount of the RNA product relative to the presence or amount of control RNA product yielded from contacting the physiological sample from a mammal that is not infected with a positive strand RNA virus under conditions that result in *de novo* initiation of RNA synthesis.

The invention further provides a method to inhibit or reduce viral infection or replication. The method comprises administering to a mammal infected with, or suspected of being infected with, a positive strand RNA virus, the genome of which encodes a viral RNA-dependent RNA polymerase, an amount of an agent effective to inhibit *de novo* initiation of RNA synthesis by the viral RNA-dependent RNA polymerase.

Also provided is a method to detect viral-specific nucleic acids. The method comprises:

(a) contacting a sample suspected of having a viral-specific nucleic acid molecule with an isolated viral RNA-dependent RNA polymerase under conditions that result in *de novo* initiation of RNA synthesis so as to yield a RNA product; and

(b) detecting or determining the presence of the RNA product.

The invention further provides a diagnostic kit for detecting a viral nucleic acid present in a biological sample. The kit comprises: (i) a recombinant viral RNA-dependent RNA polymerase, and (ii) a mixture which, when

by an arrow. The sequences of hybrid proscripts, containing both ribose and deoxyribose residues, are listed below. RNA sequences are denoted by bold capital letters while DNA sequences are in lower-case letters. Proscripts containing substitutions of the 2'-OH at position -11 relative to the initiation site were constructed to determine how this functional group interacts with the BMV RdRp. The lane number containing the RdRp product generated from each proscript in the autoradiograph below is shown to the right. (B) Autoradiograph of the BMV reaction products from the hybrid proscripts. The amount of RNA synthesis from 25 nM of each proscript is shown in lanes 1-11 with the percent activity of each hybrid proscript relative to that from the -20/13 WT proscript shown below the gel. Product sizes are denoted on the side. Lane ϕ represents the products of a control reaction with no added template. Values listed represent the mean of at least five independent experiments.

Figure 3. Ribose 2'-OHs in the subgenomic promoter are not essential for stable interaction with RdRp. (A) The sequence of the -20/15 WT proscript, directing synthesis of a 15-nt product from the initiating cytidylate (arrow) is shown. Listed below this RNA construct are the sequences of various competitors all containing at WT subgenomic promoter sequence. The -20/-1 proscript contains the WT subgenomic promoter from position -20 to -1 relative to the initiation site and serves as a negative control. The concentration of competitor needed to reduce synthesis from 25 nM of the -20/15 proscript by 50% (I_{50}) are listed to the right. (B) Determination of I_{50} values for RNA and DNA subgenomic promoters. The amount of 15-nt product generated from the -20/15 RNA proscript was measured and plotted as a function of the concentration of each competitor. The identities of the competitors are shown to the right of the graph. Data points represent the mean of three independent experiments with deviations shown.

Figure 4. Minimal DNA proscripts can inhibit viral RNA synthesis *in vitro*. (A) The sequence of the -20/15 WT proscript, directing synthesis of a 15-nt product, is shown. The arrow denotes the initiation site. Listed below are the sequences of various DNA inhibitors with successive 5' truncations. Each of the oligonucleotides containing a WT initiation sequence is capable of directing the BMV RdRp to synthesize 13-, 8- or 6-nt products, respectively. The d(-1/13)

increasing amounts of competitor DNA. The I_{50} values are given within the boxed region.

Figure 8. Initiation of genomic positive strand RNAs directed by minus strand endscripts. A) A comparison of the 3' sequences of BMV and CMV minus strand RNAs. The non-templated guanylate added to each template is in bold letters. The initiation cytidylate is indicated by an arrow. B) The predicted secondary structures of the 3' ends of BMV RNAs 1-3, B1(-)58G, B2(-)46G, and B3(-)51G. The structure predictions were generated by the MFOLD program (Jaeger et al., 1989). C) Initiation of genomic positive strand from minus strand endscripts. RdRp reaction products were separated by 12% denaturing PAGE and visualized by autoradiography. The amount of RNA synthesis from various templates relative to B2(-)46G are on the bottom of the autoradiogram. The results presented are an average from three independent trials. The sizes of the RNA products are indicated on the side of the autoradiogram. (represents the products of a control reaction with no added template. Endscripts that have a cytidylate at the +1 position are initiation-competent and indicted by a "+" while initiation-incompetent endscripts are indicated with a "(". C2(-)G and C3(-)G are endscripts of CMV RNA2 and CMV RNA3. D) Synthesis of a 200-nucleotide genomic positive strand RNA. M denotes an RNA that generates a 203-nucleotide product. Init.- endscripts have a guanylate in place of a cytidylate at the +1 initiation site. "-G" denotes a 200-nucleotide endscript without a designed non-templated guanylate. "+G" denotes a 200-nucleotide endscript with a guanylate at the 3' end of the RNA. RNA synthesis from B2(-)200+G, B2(-)200-G, and B2(-)init.- were 100%, 27%, and 0% respectively. The results presented are an average from three independent trials.

Figure 9. The effects of nucleotide changes near the initiation cytidylate on RNA synthesis. A) Effect of nucleotide changes near the initiation cytidylate. Changes from B2(-)46G (top sequence) are indicated in bold letters. "(" indictes the absence of the 3'-most nucleotide. B) Effects of the identity of the 3' non-templated nucleotide on genomic positive strand initiation. The initiation cytidylate is indicated by an arrow. Substitutions of the 3' non-templated nucleotide is indicated in bold letters. The amount of synthesis directed by the

Essential nucleotides required for stable RdRp interaction are shown in bold letters. Upstream sequence between nucleotides 17-26 required for efficient RNA synthesis is also shown. A) Recognition of the initiating nucleotide-RdRp may occur by initial binding to rGTP as primer, followed by additional interaction(s) near the initiating cytidylate. B) Following the initial binding of the RdRp complex, interaction of sequences 5' of the initiating cytidylate may be required for initiating synthesis, or C) The 5' sequences may act like an antitermination signal to move the RdRp complex into elongation phase

Figure 13. *De novo* initiation of RNA synthesis by NS5B. A) SDS-polyacrylamide gel containing recombinant NS5B preparations used in the RNA synthesis assays. Molecular weight standards (Sigma Inc., St. Louis, MO), are in lane 1 with the mass of the polypeptides indicated on the right. The arrow points to 250 ng and 400 ng of the 75 kDa purified NS5B in lanes 2 and 3, respectively. B) Schematic of the RNAs used in the experiments in panels C-D. The names of the RNAs are on the left followed by the relevant sequences. "dd-g" indicates that the guanylate was modified to have 2′ and 3′ hydrogens, while "r-g" indicates that the 3′ terminal guanylate contains a normal ribose. The 3′ guanylate, indicated by a lower case g, was added to the BVDV genomic (+)-strand RNA sequence. Nucleotides in bold are ones changed from the wild-type BVDV RNA sequence. C) Autoradiogram of a denaturing 20% polyacrylamide-urea gel demonstrating that the RNA synthesized by NS5B is initiated from the +1 cytidylate. RNAs used in the reaction are indicated above each lane. The positions of the 21-nt and 14-nt molecular weight markers used in this experiment are indicated to the right of the autoradiogram. The symbol ϕ denotes a reaction performed in the absence of exogenous templates. D) Autoradiogram quantifying the amount of RNAs produced by *de novo* versus elongative modes of RNA synthesis. E) Initiation of RNA synthesis by NS5B requires high concentrations of a guanine nucleotide. RNA (-)21g, containing a dideoxyribose in the 3′-terminal guanylate, was used in lanes 1-4 and lane 6. RNA +1C/G was used to demonstrate that the NS5B products are initiated from the +1 cytidylate. Reactions supplemented with GDP, GMP, or GTP are noted above the autoradiogram.

transcriptionally active open complex, synthesis of the first phosphodiester bond, abortive RNA synthesis, promoter clearance, processive elongation and termination. The progression of these steps is accompanied by increases in the affinity of the interaction between the polymerase and the template, with commitment of the polymerase to the template taking place during/soon after the first translocation step. The committed polymerase is thought to remain stably associated with the template even though additional nucleotides needed for elongation may be lacking in the reaction.

Viral RNA replication is mediated by RNA-dependent RNA polymerases (RdRp). For a positive-sense RNA virus, the genomic (+)-strand RNA serves as a template for synthesis of (-)-strand RNA which, in turn, serves as a template for synthesis of additional copies of genomic (+)-strand RNA and, in many viruses, (+)-strand subgenomic RNAs.

Elucidating the details of RNA synthesis by RdRp may provide the foundation for studies i) of RNA repair and recombination, ii) that identify specific inhibitors of steps in RNA synthesis by RdRp, and iii) which allow a comparison to RNA synthesis by DdRp. Results from previous characterization of *in vitro* RNA synthesis by the brome mosaic virus (BMV) RdRp defined several steps, including: (1) initiation of RNA synthesis at the penultimate cytidylate at the 3′ end of BMV (+)-strand templates (Miller et al., 1985; Kao and Sun, 1996), (2) abortive oligoribonucleotide synthesis (Sun et al., 1996), and (3) processive RNA synthesis (Sun and Kao, 1997). Steps in RNA synthesis by RdRp appear to mirror those seen in transcription by DdRps, including the release of abortive initiation products and the progression to elongation after the synthesis of nascent RNAs of 8 to 10 nt. This is perhaps not surprising since the catalytic subunits of all polymerases share common structural and functional motifs.

Despite the overall similarities in RNA synthesis by DdRps and RdRp, several differences should be mentioned. First, RdRp usually initiates RNA synthesis from the ends of RNA templates rather than exclusively from a promoter within a DNA molecule as does DdRp (Miller et al., 1986; Ishihama and Nagata, 1988; Kao and Sun, 1996). Second, RdRp appears to dissociate from the template during the abortive initiation step (Sun and Kao, 1997),

Sources of Nucleic Acids Encoding a Viral RNA-Dependent RNA Polymerase

Sources of nucleotide sequences from which the present nucleic acid molecules encoding a viral RdRp can be derived include nucleic acid from any virus preparation, including virions and cells known or believed to be naturally or experimentally infected with the virus, from which nucleic acid molecules can be isolated by methods well known to the art.

In particular nucleic acids can be isolated from viral preparations of, or cells infected with, single strand RNA viruses, preferably RNA viruses having a (+)-sense genomic RNA, e.g., Picornviridae, Caliciviridae, Astroviridae, Togaviridae, Flaviviridae, and Coronaviridae. More preferably, the nucleic acids can be isolated from viral preparations of, or cells infected with, flaviviruses, e.g., dengue virus, Japanese encephalitis virus or yellow fever virus, pestiviruses, e.g., bovine viral diarrhea virus, border disease virus, or hog cholera or classical swine fever virus, or hepatitis C viruses.

Isolation of a Gene Encoding a Viral RNA-Dependent RNA Polymerase

A nucleic acid molecule encoding a viral RdRp can be identified and isolated using standard methods, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1989). For example, reverse-transcriptase PCR (RT-PCR) can be employed to isolate and clone a viral RdRp cDNAs. A primer complementary to a sequence at the 3' end of the open reading frame encoding the viral RdRp or the 3' end of the genomic viral RNA can be employed as a primer in a reverse transcriptase reaction to prepare first-strand cDNAs from isolated RNA which contains RNA sequences of interest, e.g., total RNA isolated from infected tissue. RNA can be isolated by methods known to the art, e.g., using TRIZOL™ reagent (GIBCO-BRL/Life Technologies, Gaithersburg, MD). Resultant first-strand cDNAs are then amplified in PCR reactions.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers comprising at least 7-8 nucleotides. These primers will be identical or similar in sequence to opposite strands of the

been derived or isolated from any appropriate source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a viral genome from which the recombinant nucleic acid has been isolated. An example of preselected DNA "derived" from a viral source, would be a DNA sequence that is identified as a useful fragment within a given virus, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a DNA sequence that is prepared by RT-PCR and then excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., cloned or amplified, for use in the invention, by the methodology of genetic engineering.

Thus, recovery or isolation of a given fragment of DNA from a restriction digest can employ separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. See Lawn et al., Nucleic Acids Res., 9, 6103 (1981), and Goeddel et al., Nucleic Acids Res., 8, 4057 (1980). Therefore, "preselected nucleic acid" includes completely synthetic nucleic acid sequences, semi-synthetic nucleic acid sequences, nucleic acid sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof.

As used herein, the term "derived" with respect to a RNA molecule means that the RNA molecule has complementary sequence identity to a particular RNA or DNA molecule. Similarly, the term "derived" with respect to a DNA molecule means that the DNA molecule has complementary sequence identity to a particular RNA or DNA molecule.

Chimeric Expression Cassettes

To prepare expression cassettes for transformation herein, the recombinant or preselected DNA sequence or segment may be circular or linear, double-stranded or single-stranded. Generally, the preselected DNA sequence or segment is in the form of chimeric DNA, such as plasmid DNA, that can also

translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

The preselected DNA to be introduced into the cells further will generally contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of transformed cells from the population of cells sought to be transformed. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a co-transformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide-resistance genes, such as *neo, hpt, dhfr, bar, aroA, dapA* and the like. See also, the genes listed on Table 1 of Lundquist et al. (U.S. Patent No. 5,848,956).

Reporter genes are used for identifying potentially transformed cells and for evaluating the functionality of regulatory sequences. Reporter genes which encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene which is not present in or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Preferred genes include the chloramphenicol acetyl transferase gene (cat) from Tn9 of *E. coli*, the beta-glucuronidase gene (gus) of the *uidA* locus of *E. coli*, and the luciferase gene from firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

The general methods for constructing recombinant DNA which can transform target cells are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA useful herein. For example, J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (2d ed., 1989), provides suitable methods of construction.

plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence.

To confirm the presence of the preselected DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; and "biochemical" assays, such as detecting the presence or absence of a particular viral RdRp, e.g., by immunological means (ELISAs and Western blots).

To detect and quantitate RNA produced from introduced preselected DNA segments, RT-PCR may be employed. In this application of PCR, it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique demonstrates the presence of an RNA species and gives information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the preselected DNA segment in question, they do not provide information as to whether the preselected DNA segment is being expressed. Expression may be evaluated by specifically identifying the peptide products of the introduced preselected DNA sequences or evaluating the phenotypic changes brought about by the expression of the introduced preselected DNA segment in the host cell.

Viral RNA-Dependent RNA Polymerases

The present invention provides an isolated, purified viral RdRp, which can be prepared by recombinant DNA methodologies. The general methods for isolating and purifying a recombinantly expressed polypeptide from a host cell are well known to those in the art. Examples of the isolation and purification of such polypeptides are given in Sambrook et al., cited *supra*. Moreover, once the sequence of a particular RdRp is known, it or bioactive variants thereof can also

The invention will be further described by the following Examples.

Example 1

Subgenomic Initiation by BMV RdRp

Vast evidence supports the notion that the evolution of RNA likely had an important role during the formation of cellular life. In this "RNA world", RNA molecules are thought to have performed catalytic, as well as genomic functions (Gilbert, 1986). Examples supporting this hypothesis include: 1) the discovery of the catalytic activity found in the RNA moieties from both group I and II introns and eubacterial RNase P (Kruger et al., 1982); 2) the requirement for RNA in many essential, and presumably ancient, cellular processes such as translation, splicing, and priming of DNA synthesis; 3) the presence of ribonucleotides or derived components thereof in most biological coenzymes; 4) the biosynthesis of deoxyribonucleotides by the reduction of ribonucleotides rather than a *de novo* pathway. In order to overcome the problem of efficiently and accurately copying genetic material, it has been postulated that one of the earliest proteins would have been an RNA replicase (Lazcano et al., 1988). As DNA was eventually selected as the preferred carrier of genetic information, the pre-existing RNA-dependent RNA polymerase (RdRp) likely evolved to fulfill the new function of replicating DNA genomes, in addition to generating messenger RNAs for protein synthesis.

This proposed line of development implies a common ancestor for all polynucleotide polymerases. Fundamental similarities in the three-dimensional structure and the basic mechanism of phosphoryl transfer in the four classes of polymerases, based on whether the template and synthesized product are DNA or RNA, support this view (Steitz, 1998). It has also been argued that an important vestige of the original RNA replicase is evolutionarily conserved in the modern day eubacterial β′ subunit of DNA-dependent RNA-polymerase (DdRp) and its homologues in archeal and eukaryotic polymerases (Allison et al., 1988). Observations that both single- or multi-subunit polymerases can be induced, by mutation or change in reaction conditions, to recognize and utilize an "unnatural" template or substrate is consistent with the proposed conservation (Biebricher et al., 1996). Viral RdRps are the only extant class of polymerases that recapitulate the replication requirements of the RNA world. Presently, they

[α-^{32}P]CTP (Amersham). Reactions were incubated at 30°C for 90 minutes and stopped by phenol/chloroform extraction followed by ethanol precipitation in the presence of 5 µg of glycogen and 0.4 M ammonium acetate. Products were separated by electrophoresis on 20% denaturing (8 M urea) polyacrylamide gels. Gels were wrapped in plastic and exposed to film at -80°C. Product bands were quantified using a Phosphorimager (Molecular Dynamics). As judged from T7 DdRp generated size markers, the predominant RdRp product was 14 nts due to the nontemplated addition of one residue, a phenomenon common to many polymerases (Figure 1, lanes 1 and 10).

The all DNA proscript, designated d(-20/13), inserted deoxyriboses in every position while still containing otherwise WT subgenomic promoter and template sequences. This construct was able to direct RNA synthesis by RdRp (Figure 1, lanes 2-8); however, the predominant product was now 13 nts rather than 14 nts as it was with the RNA template. This change may reflect the need for 2'-OHs in the template in order to efficiently add the nontemplated nucleotide. The observed RNA synthesis was abolished by pretreating the d(-20/13) proscript with DNase I; however, treatment after synthesis did not degrade the RNA product (Figure 1, lanes 3 and 4). In contrast, the RdRp product was completely degraded by RNase A (Figure 1, lane 5).

Accurate initiation was verified in a number of ways: comparisons of the product sizes to those form the -20/13 WT proscript (Figure 1, lane 1 vs. 2), RNase T1 digestion (which cleaves after the initiating guanylate) resulted in labeled products 1 nt smaller than those without digestion (Figure 1, lane 6 vs. 7), the absolute requirement for GTP which is only needed for initiating accurate synthesis (Figure 1, lane 8), and the lack of synthesis from a proscript with a mutant initiation site (Figure 1, lane 9). Although the amount of RNA synthesis was approximately 6% relative to that from the WT RNA proscript, these results conclusively demonstrate that the BMV RdRp was able to productively interact with this DNA construct. The fact that RNA synthesis was obtained under identical experimental conditions as synthesis from the RNA proscript supports the idea that a primitive RdRp could have evolved to use a DNA template.

In order to copy the entire genomic template during viral replication, RdRp initiates RNA synthesis from the penultimate cytidylate of each of the

synthesis (20%) relative to the d(-20/13) proscript (6%). However, synthesis was still below that obtained form the -20/13 WT proscript (Figure 2, lane 1 vs. 2), indicating a preference for ribose residues in the template portion of the proscript. Replacement of the deoxyuridines with deoxythymidines, containing a bulky methyl group at the C5 position of the base, in the template from positions +2 to +13 in hybrid H4 did not appreciably alter RNA synthesis relative to the H1 proscript (Figure 2, lane 5).

RNA synthesis comparable to that from H1 were observed from hybrids that contained an increasing amount of deoxyriboses within the subgenomic promoter. Hybrid H2 extended the region of deoxyribose replacement, confirming that riboses at positions +1 and +2 were not important for RNA synthesis (Figure 2, lane 2 vs. 3). The H3 proscript which contains deoxyriboses at every position except those at -17, -14, -13, -11, +1, and +2 relative to the initiation site directed a similar amount of RNA synthesis as that from the H1 proscript (Figure 2, lane 2 vs. 4). The results from hybrids H2 and H3 indicated that ribose residues in the subgenomic promoter may only be required at positions -17, -14, -13, -11 or a subset thereof. However, the individual replacement of deoxyguanosine at position -17 in an otherwise RNA proscript had no effect on RNA synthesis, but the same replacement at position -11 reduced synthesis by over half relative to the -20/13 WT control (Figure 2, lanes 6 and 7, respectively). These results identified the ribose at position -11 as being important for RNA synthesis.

RdRp could recognize the 2′-OH of the ribose at position -11 by a hydrogen bond interaction. Alternatively, a change in sugar conformation resulting from the deoxyribose replacement may disrupt RdRp interaction with the base-functional groups previously determined to be essential at this position or surrounding nucleotides. To determine the role of the 2′-OH at position -11, DNA proscripts of WT sequence were synthesized with various C2′ substitutions. Chemical synthesis of the proscripts containing base analogs were performed on a ABI 394 automated DNA synthesizer (ABI, Foster City, CA) using conventional phosphoramidite elongation cycles according to Wincott et al. (1995). After subsequent ethanolic ammonium hydroxide and triethylamine trihydrofluoride treatment to cleave the exocyclic amino and 2′-OH protecting

fold reduction in I_{50} value was surprising given that d(-20/13) was reduced in the ability to direct RNA synthesis by over 15-fold relative to that from -20/13 WT proscript. The presence of either a -OH or -OMe group at the C2′ position of the -11 guanylate in an otherwise all deoxyribose proscript virtually restored the ability to be bound by RdRp, as indicated by I_{50} values of 30 nM (Figure 3). The fact that the same level of binding was observed with the 2′-OMe substitution as was seen with 2′-OH again strongly argues that this increase is not a result of simply gaining a hydrogen bond contact and points to a more subtle role for this group at position -11. As a negative control, a ribose proscript containing the WT sequences from position -20 to -1 was not able to effectively inhibit 15-nt synthesis in the range of competitor tested (10-fold molar excess).

The results obtained with the constructs in Figure 1 suggested that DNA constructs of minimal lengths could be used as potential inhibitors of viral synthesis. DNA inhibitors, containing a WT initiation sequence beginning at position -1 with increasing truncations on their 5′ ends, were tested in template competition assays. As expected, all constructs were found to direct 13-, 8- or 6-nt RNA products. All of these constructs also effectively reduced synthesis from the RNA -20/15 proscript in a manner dependent on the length of the 5′ sequence (Figure 4). As a negative control, the d(-1/13) Rev proscript which does not contain the WT initiation sequence was not able to inhibit synthesis over the range of inhibitor tested (500-fold molar excess). The sequence-specific reduction of viral RNA synthesis *in vitro* by relatively stable DNA inhibitors should allow the rational design of viral therapeutics.

Thus, RdRp has the ability to recognize and initiate accurate RNA synthesis from either an internal or terminal initiation site on a DNA template. Moreover, the functional and binding data from chemically synthesized proscripts support the conclusion that no riboses are directly contacted by RdRp during the initiation of RNA synthesis. However, it seems that riboses in the template portion of the proscript (positions +3 to +13) are needed to direct WT levels of RNA synthesis, perhaps by stabilizing the conformational change in the polymerase as it translocates on the template (Sun and Kao, 1997a; Sun and Kao, 1997b). This preference for template riboses is not observed in the minimal proscripts initiating synthesis from the penultimate nucleotide since the lengths

(v/v) Triton X-100, 2 mM $MnCl_2$, 200 μM ATP, 500 μM GTP, 200 μM UTP, 242 nM [(-^{32}P]CTP (400 Ci/mmol, 10 mCi/mL, Amersham), the desired amount of template and 5-10 μl RdRp. Following incubation for 90 min at 30(C, the reaction products were extracted with phenol/chloroform (1:1, v/v) and precipitated with six volumes of ethanol, 10 μg glycogen, and 0.4 M final concentration of ammonium acetate.

Products from RdRp reactions were digested with 2.5 units S1 nuclease (Promega) in the manufacturer's buffer for 10 min at 30(C. Denaturing loading buffer [45% (v/v) deionized formamide, 1.5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, and 0.04% (w/v) xylene cyanol] was added to the S1-treated products and denatured by heating at 90(C for 3 min prior to analysis by denaturing PAGE on 10% acrylamide gels. All gels were exposed to film at -80(C and the amount of label incorporated into newly synthesized RNAs was determined with a phosphorimager (Molecular Dynamics).

Results

DNA templates for RNA synthesis

While RNA is the preferred template for the brome mosaic virus RNA-dependent RNA polymerase, accurate initiation of RNA synthesis can take place from DNA templates. Initiation of RNA synthesis from a DNA template can take place from either a penultimate cytidylate or from an internal cytidylate in processes resembling the synthesis of genomic positive strand and subgenomic RNAs. Furthermore, the interaction between RdRp and DNA, as measured by template competition assay, is remarkably similar to interaction with RNA. The ease of manipulation of DNA templates through standard chemical synthesis makes DNA an attractive model for determining the requirements for RNA synthesis from RNA templates.

To examine synthesis from DNA templates, a deoxyoligonucleotide named d(−1/13) was used as the prototype. d(−1/13) contains the sequence complementary to nucleotides 1241 to 1252 of BMV RNA3. The 3'-most nucleotide is a guanylate, which along with the initiation cytidylate (+1), has previously been demonstrated to be necessary for RNA synthesis. Two guanylates were added at the 5' end to allow transcription initiation by T7 polymerase and direct the incorporation of radiolabeled cytidylates.

and a change of +6 U/G resulted in 40% synthesis. A change of +7 A/G also resulted in wt d(−1/13) level of RNA synthesis (data not shown). These results suggest that the incorporation of a guanylate in the first four nucleotides of the nascent RNA will decrease the efficiency of RNA synthesis, but that the requirement is relaxed starting at the +5 position.

Interaction between DNA template and RdRp

Changes at various positions along the DNA template could affect either the ability of the template to interact stably with RdRp or the efficiency of nucleotide incorporation into the nascent RNA. A template competition assay was used (Siegel et al., 1997) to address these two possibilities. The reaction contains limiting amount of RdRp relative to the template concentration, and a reference RNA, r(-20/15) which directs the synthesis of a 15-nt product. Many of the competitors chosen for this analysis were found to debilitate RNA synthesis and synthesize negligible amounts of product (Figure 6). For simplicity the results described below will be from the reference of r(−20/15). Competitor wt d(−1/13) effectively reduced RNA synthesis to 60% when present at five-fold excess and its effect was more severe at ten-fold excess. Removal of the 3'-most nucleotide, d(+1/13) resulted in a DNA template which can no longer compete effectively, RNA synthesis was found to be greater than 90% even at five and ten-fold molar excess (Figure 6). The ability to inhibit RNA synthesis was partially restored when the 3'-most nucleotide of the template was a cytidylate, consistent with this template being a more effective template. When the initiation nucleotide was at the fourth position from the 3' end due to the addition of 3' AAG, the ability to inhibit synthesis was again reduced.

The initiating cytidylate is also involved in the stable interaction with RdRp. At five molar excess, a change of the cytidylate to a guanylate (+1C/G) reduced its ability to stably interact with RdRp. A significant decrease in synthesis at ten molar excess suggests that the ability to interact is partially retained. Also, previous studies with the BMV subgenomic RNA promoter showed that a change of the initiation nucleotide was able to reduce, but not abolish stable interaction with RdRp. Furthermore, substitution of specific nucleotides in the subgenomic promoter suggests that the interaction with RdRp at the initiation site requires the primer nucleotide, GTP (Siegel et al., 1997).

In light of the observations made with DNA templates, the requirements for the initiation of BMV genomic positive strand RNA synthesis were examined. Short RNAs of 58-, 46-, and 51-nucleotides corresponding to the minus strand of BMV RNA 1, 2, and 3 respectively, with an extra guanylate at the 3' end were generated. These short transcripts were termed "endscripts" as they represented the minus strand 3' ends of BMV. All three endscripts have complete stem-loop structure observed to be required for positive strand genomic RNA synthesis *in vivo* (18) (Figure 8B). After a standard RdRp reaction, the products were analyzed on a denaturing PAGE. Endscripts B2(-)58G, B2(-)46G, and B3(-)51G were all able to direct positive strand synthesis with comparable efficiency (Figure 8C lanes 1-2, 5-6, and 9-10). A high resolution gel shows that these bands my be composed of two distinct sizes, possibly due to non-templated terminal nucleotide addition by the BMV RdRp (Siegel et al., 1997). As a control, endscripts of cucumber mosaic virus (CMV) minus strand of CMV RNA 2 and 3, termed CMV2(-)G, and CMV3(-)G were tested. CMV2(-)G of 64 nucleotides was able to direct synthesis at only 10% of B2(-)46G, and CMV3(-)G endscript of 54 nucleotides was unable to direct any discrete RNA products (Figure 8C, lanes 13-16). The synthesis from BMV endscript is thus species specific. To determine that initiation took place from the penultimate cytidylate, the +1 cytidylate was mutated to a guanylate in all three BMV endscripts. As expected, endscripts generated from these initiation mutants were much reduced to direct positive strand synthesis (Figure 8C, lanes 3-4, 7-8, and 11-12), indicating that genomic positive strand RNA synthesis is initiated from the cytidylate presumed to be used *in vivo*. A smeared RNA product is observed in lanes with B3(-)51G initiation mutant endscripts (Figure 8C lanes 11-12). This smeared product could be due to endlabelling of the input template. In comparison to the synthesis of a 46-nt RNA from the subgenomic promoter (Adkins et al., 1997), synthesis of genomic positive strand RNA is reproducibly a third per pmole of input template. However, these results clearly indicate that *in vitro* BMV RdRp is able to specifically distinguish BMV promoters and initiate genomic positive strand synthesis, and perhaps regulate different levels of RNA synthesis.

most end. Following a standard RdRp reaction, the products were analyzed on a denaturing PAGE. Consistent with the results from DNA templates the absence of a non-templated nucleotide at the 3' end significantly reduced RNA synthesis (Figure 9A, lane 2). The 3'-most guanylate could be substituted with a uridylate, adenylate or cytidylate with only moderate reductions to the levels of positive strand synthesis (Figure 9B, lanes 1-4). These results indicate that the 3'-most non-templated nucleotide needs to be present for efficient RNA synthesis. Although the presence of a guanylate at this position directed synthesis most efficiently, the identity of the nucleotide at this position is not absolutely crucial. However, the addition of two nucleotides (AG) or three nucleotides (AAG) 3' of the initiating cytidylate reduced positive strand RNA synthesis to less than 15% of wildtype. These results indicate that in RNA templates the BMV RdRp prefers the initiating cytidylate to be at the penultimate position.

Changes in the template could either affect the stability of interaction with RdRp and/or the ability of RdRp to direct synthesis. To distinguish between these two possibilities, the competition assay described above was employed. As a control, a 53-nt RNA unrelated to BMV (PCRII/53) was used as non-specific competitor. PCRII/53 caused a slight decrease in synthesis from the reference RNA r(–20/15) to 66% when present at 250 nM, the highest concentration tested, suggesting that there is limited amount of non-specific RdRp-RNA interaction (Table 1). The B2(-)46G endscript was more effective competitor and reduced synthesis from r(–20/15) with an I_{50} value of 120 nM (Table 1). This value is similar to the one obtained for the DNA template, d(-1/13) (Figure 7). Removal of the -1 nucleotide at the 3' end ((-1), or changing the +1 cytidylate to a guanylate (+1C/G) decreased the competitiveness of these RNAs (Table 1) and inhibition of synthesis never reached below 50% even at the highest concentration tested. Therefore, the I_{50} value for these RNAs are listed as (250 nM. Changes at the +2 position (+2A/G) also resulted in a slight reduction in the template's ability to interact with the RdRp complex, but not to the degree observed for –1 or +1 positions (Table 1). Changes at the +3 position (+3U/A) yielded an I_{50} value similar to wt B2(-)46G (Table 1). Competition assays using CMV2(-)G and CMV3(-)G endscripts showed that BMV RdRp does bind to CMV minus strand templates (24). This is not surprising since the

TABLE 1. Mutations in the endscript affect the ability to interact with RdRp.

Construct	3′ Sequence	I_{50}(nM)
B2 (-) WT	3′ **G**CAUUU . . . N46 5′	120
B2 (-) Δ -1	3′ -CAUUU . . . N46 5′	>250
B2 (-) Δ-1,+1,+2	3′ --UUU. . . N44 5′	>250
B2 (-) +1 C/G	3′ **GG**AUUU . . . N46 5′	>250
B2 (-) +2 A/G	3′ **GCG**UUU . . . N46 5′	235
B2 (-) +3 U/A	3′ GCA**A**UU . . . N 46 5′	140
Non-specific RNA	3′ AUAGGU . . . N53 5′	>250

(Figure 11B lanes 7-8 and 11-12). The high amount of RNA synthesis from templates with the L1 deletion was likely due to replacement of the L1 loop region with 5' sequences as we shall demonstrated below.

To further determine the effect of 5' sequences, endscripts with truncations at the 5' end were made and tested. When the template sequence was reduced to 26-nucleotides in B2(-)26G, it was still able to direct efficient positive strand RNA synthesis (Figure 11C lanes 4-5). However, a further deletion of four and ten nucleotides in endscripts B2(-)22G and B2(-)16G, reduced RNA synthesis to 22% and 5% respectively (Figure 11C lanes 10-11 and 13-14). These results suggest that nucleotides 17-26 may be required for efficient synthesis. To confirm this without changing the length of the RNAs, transversion of nucleotides at positions 17-24 in the context of the 26-mer (B2(-)26 TV) were tested and found to result in only 17% synthesis in comparison to B2(-)26G (Figure 11C lanes 7-8). In all cases, initiation took place from the authentic +1 cytidylate, since a mutation at the initiation cytidylate failed to direct RNA synthesis (Figure 11C lanes 3, 6, 9, 12, and 15). The results identify nts 17-26 as being crucial for RNA synthesis.

The internal deletion of nucleotides 17-26 in the context of B2(-)46G reduced synthesis to 70% of wt. Whereas, transversion of the same sequence in the context of B2(-)26G reduced synthesis to 17% of wt. This incongruity in the observed results prompted the examination of the sequence present in the nucleotides 17-26 region more closely (Figure 11D). All of the endscripts contain two 5' guanylates. These two Gs are natural in BMV sequence at these positions in B2(-)46Gand B2(-)26G and are brought into this position from upstream sequences in the deletion constructs B2(-)(3-11 and B2(-)(17-26 (Figure 11D). In the B2(-)26TV construct the two Gs were placed in to allow transcription by T7 polymerase. In addition two adenylates are present at approximately the same position four or five nts 3' from the guanylates (Figure 11D). RdRp displays a certain amount of flexibility in adjusting to minor spatial perturbations that may allow the recognition of the two adenylates in B2(-)(17-26 . A transversion of nucleotides 17-26 resulted in the two adenylates being placed at only one nucleotide from the 5' guanylates (Figure 11D).

templates generated by T7 RNA polymerase tends to add extra nucleotides at the terminal end (Cazenave et al., 1994). Also, the use of DNA templates which are easily modified and less expensive to generate could be used as potential templates to study the requirements for recognition and synthesis by RdRp.

RNA synthesis from the minus strand RNA endscripts was about a third as productive in comparison to synthesis directed by a subgenomic promoter. The amount of RNA synthesized from the three BMV promoters from more efficient to least as: subgenomic RNA > genomic RNA > minus strand RNA.

Similar to observations with DNA templates, initiation of BMV genomic positive strand synthesis from RNA templates was found to take place efficiently only in the presence of the non-templated nucleotide at the 3' end. The presence of a non-templated nucleotide at the 3' end of the minus strand has been reported in CMV and semliki forest virus (Collmer et al., 1985: Wengler et al., 1979). In addition, it has been demonstrated that the presence of the non-templated guanylate residue is required for generating CMV associated satellite RNA (CARNA5) (Wu et al., 1984). In CMV, semliki forest virus, as well as CARNA5, the extra nt at the 3' end was found to be a guanylate and the results from the *in vitro* experiments with BMV endscripts suggest that there may be a preference for a guanylate.

Terminal transferase (TNTase) activity may be common to all polymerases. In addition to BMV RdRp, other polymerases have also been shown to have TNTase activity. These include poliovirus 3D polymerase, T7 RNA polymerase, vaccinia virus, recombinant bovine viral diarrhea virus NS5B. The hepatitis C RdRp may be an exception in that the TNTase activity observed along with RdRp may be due to a cellular transferase.

RdRp-template interaction

In the absence of an extra nucleotide 3' end of the initiating cytidylate, the template was unable to direct efficient synthesis and was also a poor competitor. It is likely that the presence of the extra nucleotide at the 3' end of the initiation site provides stability to the interaction between the template and RdRp. RNA templates without the 3' non-templated nucleotide showed 25% synthesis compared to the control, whereas, synthesis from a DNA template lacking the 3' nucleotide was reduced by 90%. It is possible that a fraction of

contributions of the positive strand RNA *in vivo*, it raises the possibility that observations of Pogue and Hall's *in vivo* experiments could reflect some requirements other than those required for initiation of genomic positive strand RNA synthesis. The data further suggests that the sequence complementary to ICR2, nucleotides 17-24, is involved in RNA synthesis. Transversion of the sequence complementary to ICR2 (Pogue and Hall, 1992), in the context of a 26 nucleotide minus strand RNA or the deletion of this sequence greatly reduced the template's ability to direct synthesis. Taken together these observations suggest that the promoter sequence required for directing positive strand should be present within the first 26 nucleotide region of the minus strand RNA.

Thus, the following working model for the mechanism of genomic positive strand synthesis is proposed. There appear to be two requirements: 1) nucleotide sequences required for stable binding to RdRp, and 2) additional 5' sequences required for processive synthesis. The fact that B2(-)26TV was unable to direct efficient synthesis in comparison to B2(-)26G argues against the point that in template length may be a factor. Furthermore, the transversion and deletion analysis of L1 loop region suggest that the key sequences there and the initiation nucleotides could be recognized in a sequence specific manner (Figure 12). In the native BMV RNAs the stem L2 region may be required for presenting the sequences in the loop region to the RdRp in an appropriate conformation.

Example 3

De Novo Initiation by a Flavivirus RdRp

Materials and Methods

RdRp activity assay and product analysis. BVDV NS5B was prepared from recombinant baculovirus infected Sf9 cells as described in Zhong et al., 1998. RNAs were chemically synthesized by Oligos etc (Wilsonville, OR) and DNAs were synthesized by Operon Inc (Almeda, CA). Each RNA or DNA was purified by HPLC chromatography and the amount of purified nucleic acid determined by gel electrophoresis and Toluidine Blue staining, and by spectrophotometry. Standard assays consisted of 5 pmole of template (unless stated otherwise) with 20 ng of NS5B in a 40 µl reaction containing 20 mM sodium glutamate (pH 8.2), 4 mM $MgCl_2$, 12.5 mM dithiothreitol, 0.5% (v/v)

In a reaction containing NS5B, (-)21g directed the synthesis of two predominant products of 21- and 22-nts as judged by comparison to RNAs of known lengths (Figure 13C, lane 2, and data not shown). The 22-nt product is likely due to the above mentioned nontemplated nucleotide addition. Change of the penultimate (the +1) cytidylate to either a guanylate in template +1C/G or to an uridylate in +1C/U drastically reduced RNA synthesis (Figures 5B and 5C, lanes 3 and 4), suggesting that the +1 cytidylate is the initiation nucleotide. The detrimental effect of nucleotide substitutions was specific to the +1 nucleotide since changes of the +2 adenylate to a guanylate and a transversion of the +3 uridylate did not negatively affect RNA synthesis. However, the latter change did result in a slight alteration in the mobility and abundance (a 3- to 5-fold increase) of the 21- and 22-nt products (Figure 13C, lanes 5 and 6).

RNAs +2A/G and +3U/A also resulted in products of higher molecular weight, possibly because of the polymerase stuttering on the template or using the nascent RNA as template for additional rounds of RNA synthesis (Figure 13C, lanes 5 and 6). The lack of a ladder of bands leading up to the more predominant higher molecular weight product is more consistent with the hypothesis that these are multimeric products generated from the nascent RNA. These results indicate that *de novo* initiation of (+)-strand BVDV RNA synthesis by the recombinant NS5B protein can take place from an internal initiation nucleotide.

The relative frequency of RNA synthesis by extensions from a 3′ terminus or by a *de novo* mechanism was determined. RNA r(-)21g was made to be identical in sequence to (-)21g, except that the 3′-most guanylate contained a ribose that could provide a 3′ hydroxyl for nucleotidyl extension. As a control, r+1C/G was synthesized, in which the initiation cytidylate was changed to a guanylate. Template r(-)21g was found to direct the synthesis of RNA products indistinguishable in size from those produced by (-)21g (Figure 13D, lanes 3-5). RNA synthesis from r(-)21g is dependent on the +1 cytidylate since r+1C/G did not direct any product synthesis (Figure 13D, lanes 6-8). Therefore, *de novo* initiation seems to be the preferred mode of RNA synthesis.

The use of the penultimate cytidylate indicates that GTP is required to initiate product synthesis. Previous work has established the need for higher

Further similarities include the lack of an effect in response to a change of the +2A to a guanylate in the DNA context.

Template requirements for *de novo* initiation of RNA synthesis. Templates of minimal lengths were previously used to identify the nucleotides required for interaction with the BMV RdRp (Example 2). To probe the identity of the nucleotides and the relative positions required for initiation by NS5B, a series of RNAs with changes focused on the 3′ end was synthesized. Despite *de novo* initiation seeming to be the predominant form of RNA synthesis (Figure 5D), all of these RNAs were made with the 3′-most nucleotide containing a dideoxyribose to eliminate the possibility of polymerase extension from the 3′ end of the template. Removal of the 3′-most nontemplated nucleotide in RNA (-)21 resulted in a template that gave a seven-fold increase in the correctly initiated RNA products in comparison to (-)21g, demonstrating that initiation can efficiently take place from the 3′-most nucleotide (Figure 14, lane 2). However, there is also an increase in the amount of truncated products, suggesting that some synthesis may be abortive. To ensure that initiation took place from the 3′ end of the template, RNA (-)21+1C/G was tested, which changes the +1 cytidylate to a guanylate. Templates containing this change were unable to direct RNA synthesis (Figure 14, lane 4). Initiation of RNA synthesis by BVDV NS5B may prefer a cytidylate as the 3′ nucleotide, although a cytidylate at the penultimate position is also acceptable.

To determine whether the initiation nucleotide needs to be positioned near the 3′ end, RNAs were tested that contained two (+3′ag) or three (+3′aag) extra nucleotides 3′ of the initiation cytidylate (Figure 14A). RNA +3′ag directed ca. 5% of RNA synthesis compared to (-)21g (Figure 14, lane 5). However, +3′agg reduced product synthesis to background levels (Figure 14, lane 6). The preference for the position of the initiation nucleotide was then examined from a template (named gCCC) that has three cytidylates positioned at the +1, +2, and +3 positions. The change of the +2 and +3 nts to cytidylates would not be expected to affect RNA synthesis since previous changes at these positions did not negatively affect RNA synthesis (Figure 13C). The gCCC template was able to direct the synthesis of an array of products around 21-nts and longer.

Competitor RNAs were all tested in triplicate and at two different concentrations, 5 and 20 pmoles, i.e. at a ratio of 1:1 and 4:1 of the competitor and 3-init RNA. The amount of synthesis directed by 3-init in the absence of competitors was set as 100% (Figures 8A, 8B). RNA (-)21g at an equal molar ratio to 3-init was able to reduce synthesis from 3-init to approximately 36% (Figure 8B). Change of the initiation cytidylate to a guanylate resulted in a poor competitor, allowing 84% of the synthesis of the 3-init product observed in the absence of any competitor. Quite interestingly, a change of the initiation cytidylate to a uridylate (+1C/U) resulted in a better competitor in comparison to +1C/G, suggesting that NS5B was still able to bind to the RNA containing a uridylate at the +1 position, but was unable to efficiently direct RNA synthesis (Figure 13C, lane 4).

Extensions to the 3′ end of the template had a detrimental effect on the ability of the template to interact with NS5B. Addition of two nucleotides 3′ of the initiation cytidylate in RNA 3′ag resulted in reduced interaction with NS5B, while the addition of three extra nucleotides in 3′aag further decreased the ability to compete with 3-init for recognition by NS5B (Figures 8A, 8B). All of the trends observed in competition assays with equal molar amounts of competitor and 3-init were also observed in assays with four molar excess of competitor.

In addition, the initiation cytidylate (+1C) has several moieties needed for recognition by the RdRp. These include: all of the moieties in the base, and the phosphate connecting this cytidylate to the penultimate nucleotide. However, the ribose of the cytidylate can be change to a deoxyribose without significantly impacting RNA synthesis. The requirements for the template include a ribose from +2 to +11, while after the +12 position, the presence of a deoxyribose does not effect RNA synthesis. Also, in the template sequence (from +2 toward the 5' end), base requirements are relaxed. Modification of the base with a bulky C5 methyl group is acceptable (where it is not at the +1C). Also, H-bonding requirements in the nucleotides beyond the +1 position are relaxed (where it is not at the +1C).

Discussion

Elucidating the mechanism of RNA synthesis has potential benefits for the design and testing of antiviral compounds. RdRp is also of interest in that it

not have an initiation nt at the appropriate position to induce *de novo* initiation. In the presence of a 33-nt RNA that lacked an initiation cytidylate at the 3′ terminus, it was noticed that synthesis by BVDV NS5B was limited to the elongative mode of RNA synthesis (data not shown). Third, the initiation of RNA synthesis from the sequences directing (-)-strand RNA synthesis studied by others may be different for the initiation of (+)-strand RNA synthesis described herein. Previous work on RNA synthesis by the BMV RdRp demonstrates that there are different requirements in the templates for (-)-strand and (+)-strand synthesis (Adkins et al., 1998).

These results with recombinant BVDV NS5B can be compared and contrasted with those from studies of the BMV RdRp complex enriched from infected plants. One similarity between the BMV RdRp complex and the recombinant NS5B protein is the requirement for a template +1 cytidylate as the initiation nucleotide for RNA synthesis. Also in both systems, inefficient RNA synthesis was observed from a uridylate at the penultimate position (Pata et al., 1995, and Figure 14C). Further, both RdRps require higher GTP concentration during the initiation of RNA synthesis than they do for elongation and both are inhibited by additional sequences present 3′ to the initiation cytidylate (Pata et al., and Figure 5E).

Several other requirements for initiation are different between the BMV RdRp and the recombinant BVDV NS5B. For the BMV RdRp, initiation of RNA synthesis must take place from the penultimate nucleotide; removal of the 3′-most nucleotide abolished RNA synthesis (Siegel et al., 1998). Furthermore, the identities of the +2 nucleotide and the additional 5′ sequences appear to modulate the efficiency of RNA synthesis (Siegel et al., 1998; Adkins et al., 1998). In contrast, the BVDV NS5B protein prefers the 3′-most nucleotide for initiation as opposed to the penultimate nucleotide. It also does not seem to be affected by the identity of the +2 nucleotide, or apparently, by the upstream sequences, as indicated by the efficient RNA synthesis from RNA 3-init that was altered in the spacing between the template initiation nucleotide and the normal 5′ sequences. Whether these discrepancies should be attributed to differences in viral species or to a difference between a polymerase complex and a recombinant protein await further characterizations.

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WHAT IS CLAIMED IS:

1. A method to detect *de novo* initiation of RNA synthesis, comprising;

(a) contacting a sample comprising a nucleic acid template with isolated recombinant viral RNA-dependent RNA polymerase under conditions that result in *de novo* initiation of RNA synthesis so as to yield a RNA product; and

(b) detecting the presence of the RNA product.

2. A method to identify agents that specifically inhibit *de novo* initiation of RNA synthesis, comprising;

(a) contacting the agent with a mixture comprising an isolated recombinant viral RNA-dependent RNA polymerase and a nucleic acid template under conditions that result in *de novo* initiation of RNA synthesis so as to yield a RNA product; and

(b) detecting or determining the presence or amount of the RNA product in the presence of the agent relative to the presence or amount of control RNA product yielded from contacting a mixture comprising an isolated recombinant viral RNA-dependent RNA polymerase and a nucleic acid template under conditions that result in *de novo* initiation of RNA synthesis, wherein a reduced amount of the RNA product in the presence of the agent is indicative that the agent inhibits *de novo* initiation of RNA synthesis.

3. The method of claim 1 or 2 wherein the template is RNA.

4. The method of claim 1 or 2 wherein the template is DNA.

5. The method of claim 1 or 2 wherein the viral polymerase is a flavivirus polymerase.

6. The method of claim 1 or 2 wherein the viral polymerase is a pestivirus polymerase.

(b) detecting or determining the presence of the RNA product.

14. The method of claim 11 or 13 wherein hepatitis C virus is detected.

15. The method of claim 11 or 13 wherein the sample is from a human.

16. The method of claim 11 or 13 wherein the sample is a fluid sample.

17. The method of claim 16 wherein the fluid is blood serum, plasma, whole blood, or saliva.

18. The method of claim 11 or 13 wherein the sample is a tissue sample.

19. A diagnostic kit for detecting a viral nucleic acid present in a biological sample comprising: (i) a recombinant viral RNA-dependent RNA polymerase, and (ii) a mixture which, when combined with the recombinant viral RNA-dependent RNA polymerase and the viral nucleic acid in the sample, results in *de novo* initiation of RNA synthesis.

20. The kit of claim 19 wherein the mixture comprises nucleotides.

21. The kit of claim 20 wherein the nucleotides are ribonucleotide triphosphates.

22. The kit of claim 19 wherein the mixture comprises a modified nucleotide.

23. The kit of claim 22 wherein the modified nucleotide has a modified base.

24. The kit of claim 22 wherein the modified nucleotide has a modified ribose.

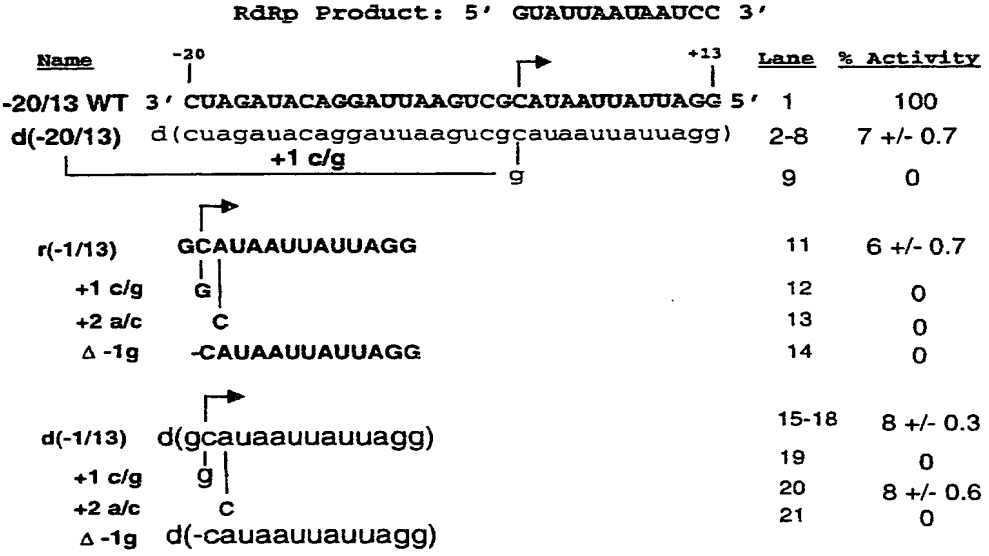

FIG. 1A

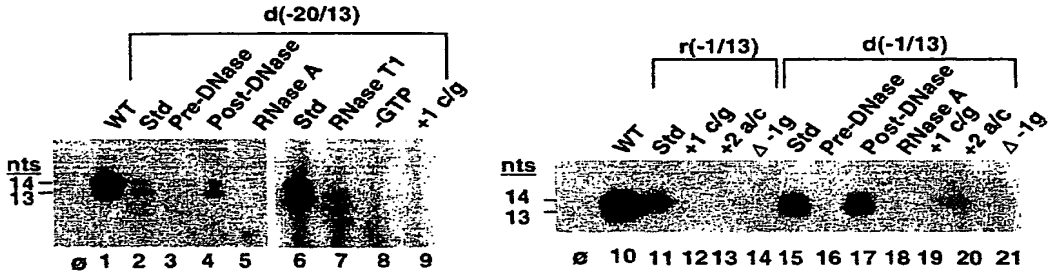

FIG. 1B

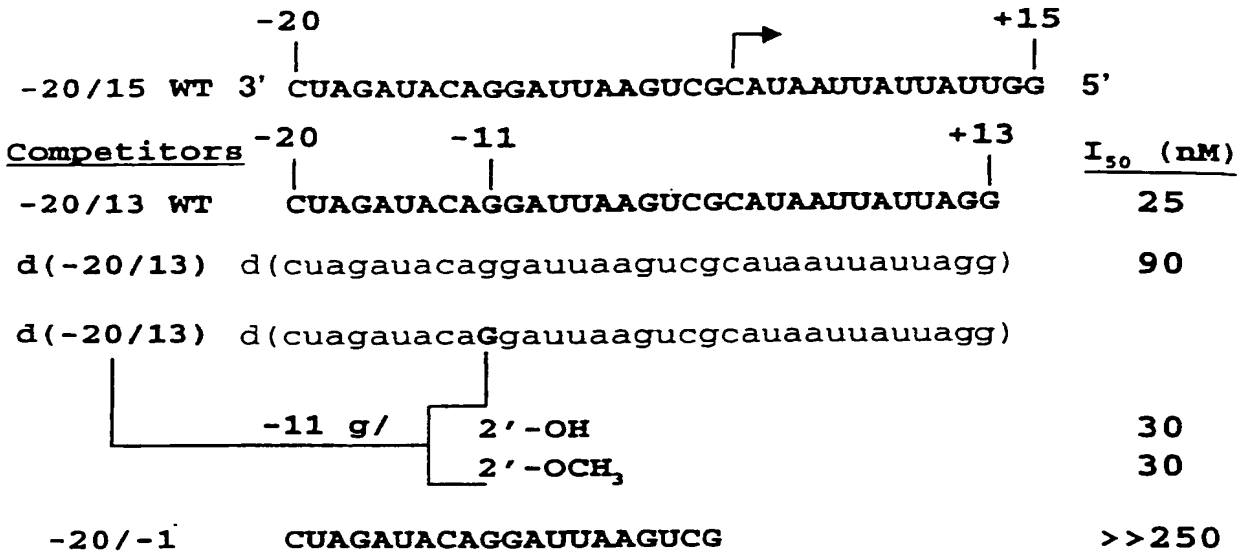

FIG. 3A

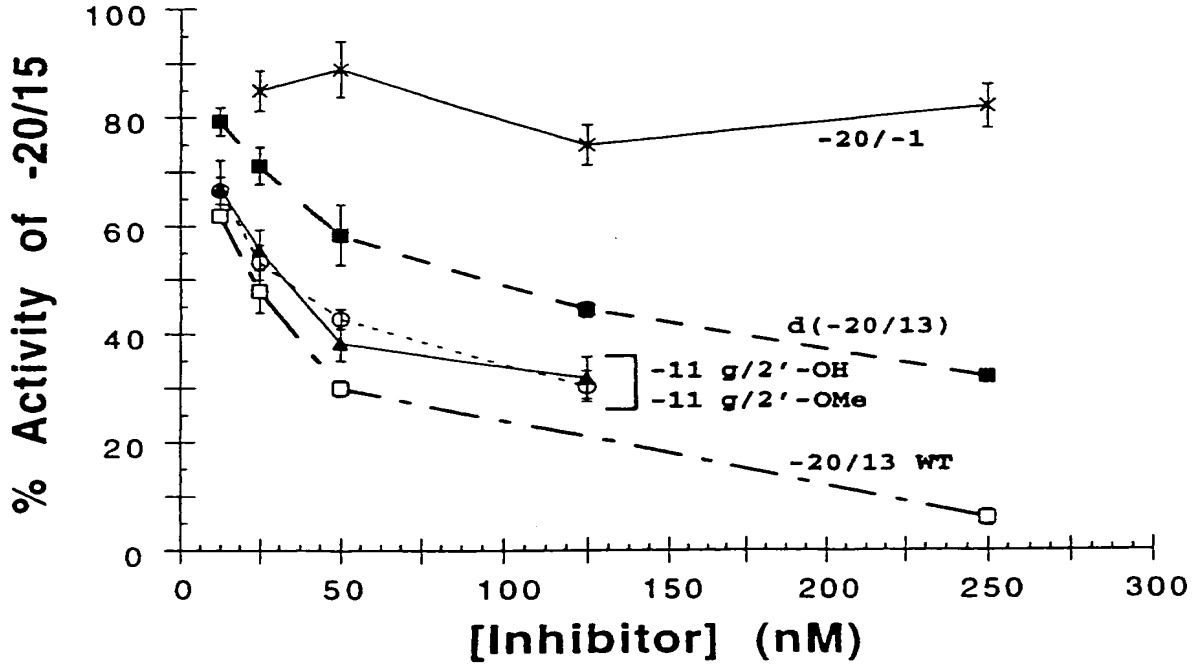

FIG. 3B

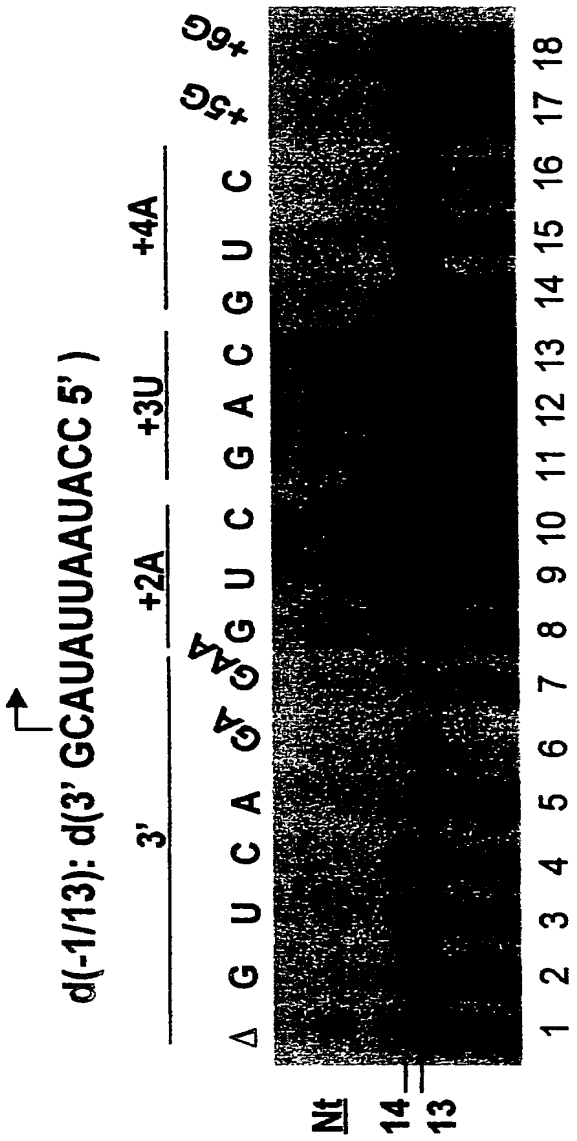

FIG. 5A

Name	Sequence	%synthesis	% synthesis from r(-20/15) 5X comp.	10X comp.
Wt	3' GCAUAUUAAUACC	100	60 +/- 12	43 +/- 6
+1/13	Δ----------	10	95+/- 10	90 +/- 2
3'C	C----------	38	75 +/- 10	ND
+GAA	AA----------	14	94 +/- 6	ND
+1C/G	--G----------	5	98 +/- 13	62 +/- .3
+2G	----G----------	29	64 +/- 12	38 +/- 1
+2U	----U----------	118	73 +/- 9	46 +/- 5
+3G	------G----------	7	82 +/- 3	58 +/- 3
+3A	------A----------	19	ND	47 +/- 1
+4G	---------G----------	21	79 +/- 5	56 +/- 5
+5G	----------G----------	110	57 +/- 9	47 +/- 3
+6G	----------G----------	40	60 +/- 2	43 +/- 4

FIG. 6

FIG. 8A

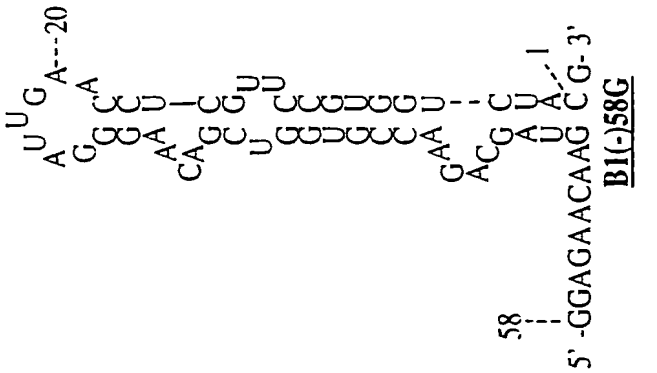

FIG. 8B

		Lane	% Syn
B2(-)46G	: GCAU..... N46	1	100
B2(-)46	: - CAU... .. N46	2	25
B2(-)46+1C/G:	GGAU.....N46	3	2
B2(-)46+2A/G:	GCGU.....N46	4	8
B2(-)46+3U/A:	GCAA.....N46	5	110

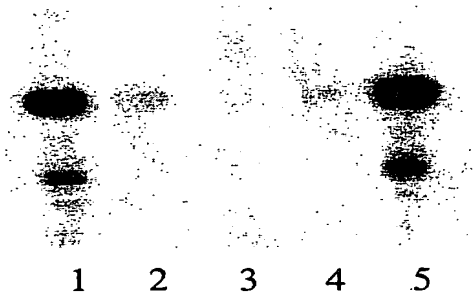

FIG. 9A

	Lane	% Syn
B2(-)46G: GCAU.....N46	1	100
B2(-)46C: CCAU.....N46	2	85
B2(-)46A: ACAU.....N46	3	40
B2(-)46U: UCAU.....N46	4	45

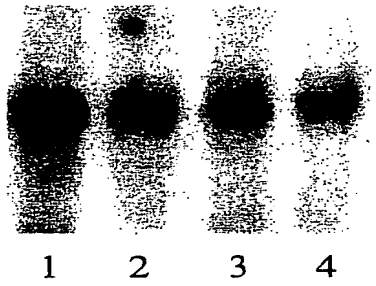

FIG. 9B

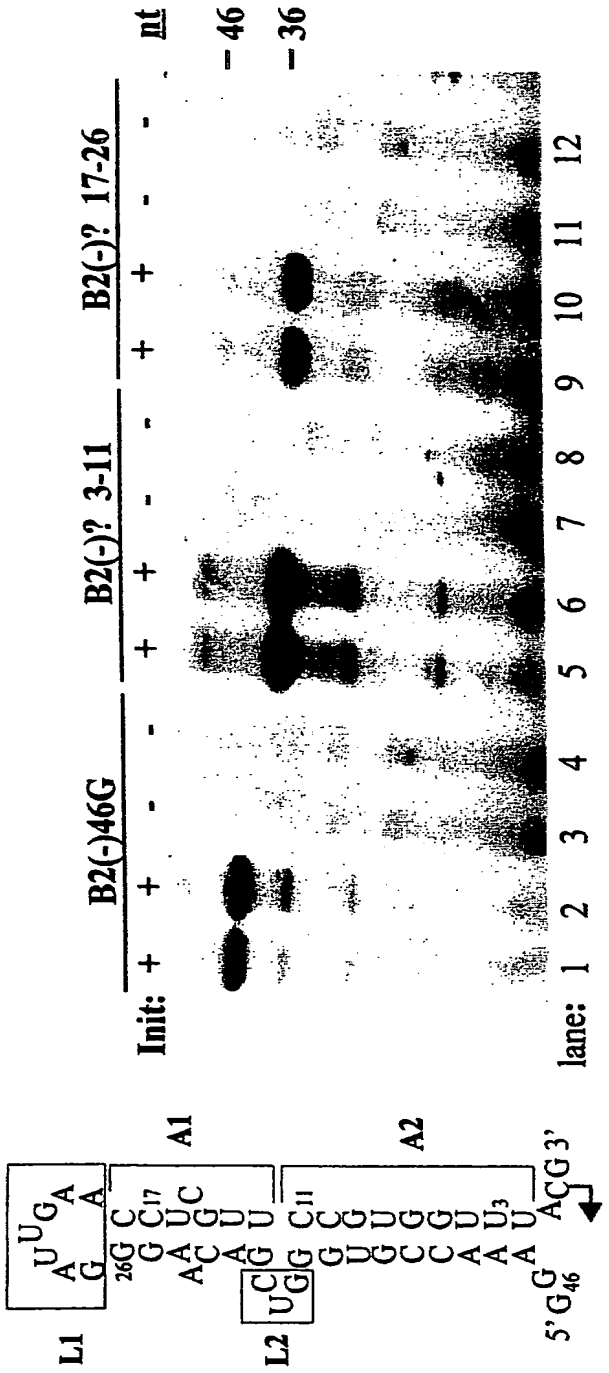

FIG. 11B

FIG. 11A

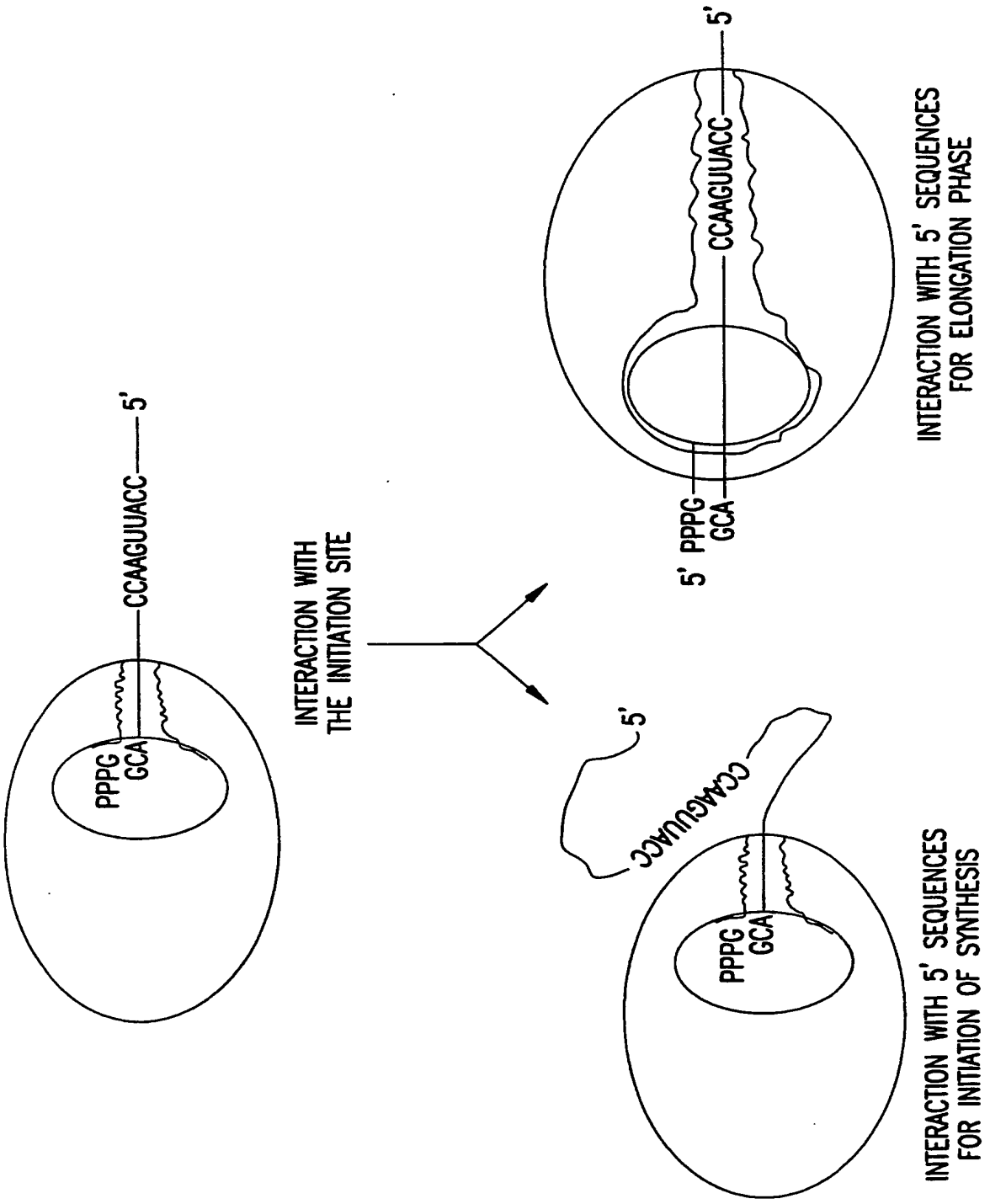

FIG. 12

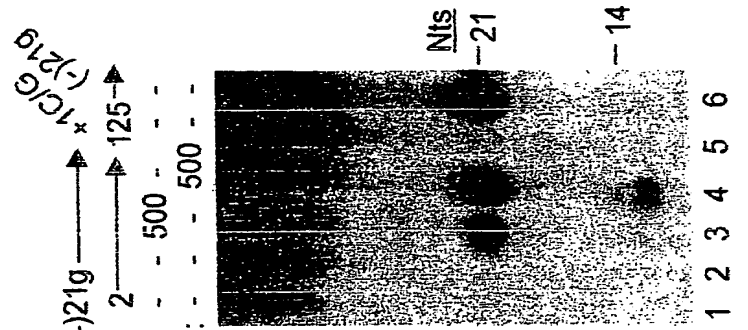

FIG. 13E

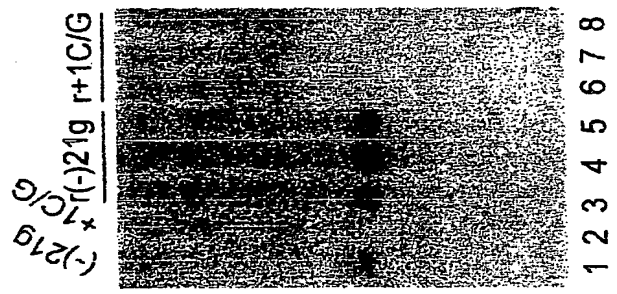

FIG. 13D

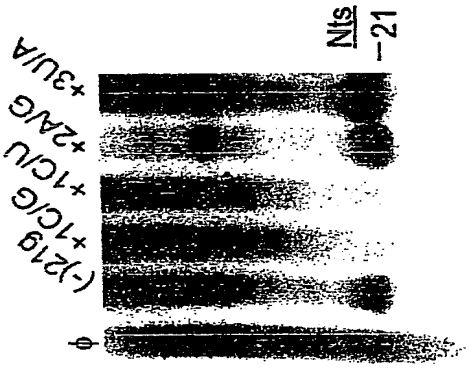

FIG. 13C

(-)21g	3'	gCAU— 5'
(-)21	3'	- CAU— 5'
(-)21,+1C/G	3'	- GAU— 5'
+3'ag	3'	agCAU— 5'
+3'aag	3'	aagCAU— 5'
3' gCCC	3'	gCCC— 5'
3' gGCC	3'	gGCC— 5'
2-init	3'	gcauCAU—5'
3-init	3'	gcaucauCAU— 5'

FIG. 15A

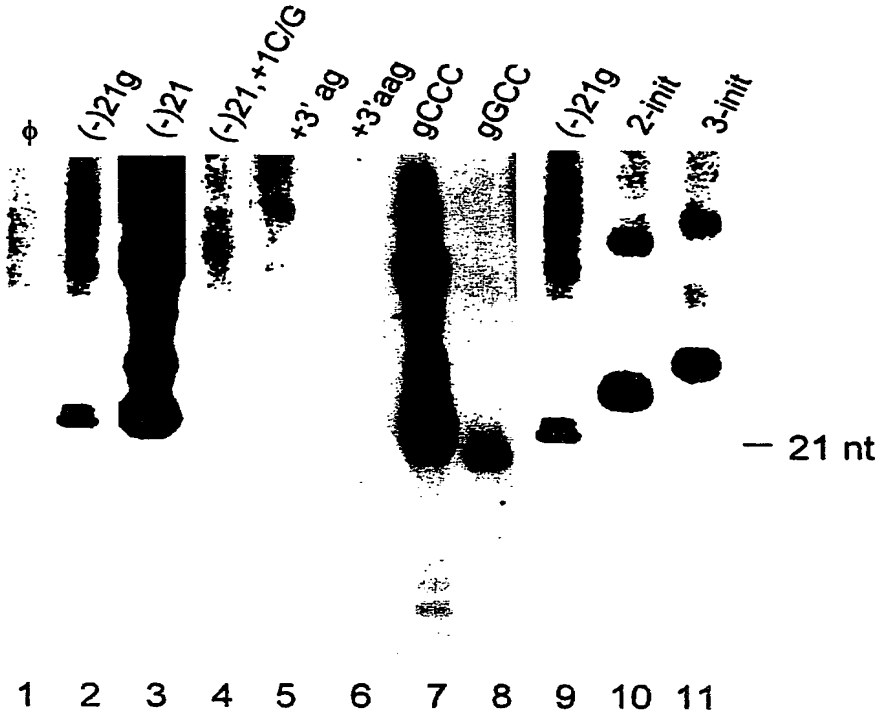

FIG. 15B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/00152

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/70 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LOHMANN V ET AL: "BIOCHEMICAL PROPERTIES OF HEPATITIS C VIRUS NS5B RNA-DEPENDENT RNA POLYMERASE AND IDENTIFICATION OF AMINO ACID SEQUENCE MOTIFS ESSENTIAL FOR ENZYMATIC ACTIVITY" JOURNAL OF VIROLOGY,US,THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 71, no. 11, November 1997 (1997-11), pages 8416-8428, XP000877461 ISSN: 0022-538X the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 July 2000	20. 07. 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/00152

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 12,14-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.